Functional Characterizations of the Reciprocal Interaction of the Circadian Clock gene *Time for coffee* (*TIC*) with Stress and Energy in Arabidopsis

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Abstract

Circadian clocks are important time-environment response mechanism that provide adjustment and adaptation for light, dark, warm, cold or wet, dry altering; some of the altering even caused stresses in plants. The *Arabidopsis thaliana* circadian clock is usually composed of input pathway, output pathway and core oscillator. In current knowledge, the core oscillator includes three negative regulation feedback loops, the loop which on the center position was composed of TOC1, CCA1 and LHY. Thus, the morning expressed genes CCA1 and LHY inhibits the transcription of the evening gene TOC1. At dusk, TOC1 expression is also promoted the degradation of the protein CCA1 and LHY; therefore, TOC1 activated the transcriptional level of morning genes. Until now, how other signals regulate this clock in the input pathway is still not clear. As previously reported, *Time for coffee* (*TIC*) knock out mutant *tic* was originally found as a significant short circadian period phenotype clock mutant. Therefore, TIC could impact time-specific activity event with the core oscillator in circadian clock. Thus, TIC and kinase AKIN10 (SNF1-related protein kinase) toward the circadian clock was genetically tested, the affection of AKIN10 to circadian clock which was TIC dependent was found. Additionally, *tic* also causes numerous developmental, metabolic, and stress-related phenotypes.

To investigate further functions of TIC in the regulation of the circadian clock, I investigated the circadian period of *TIC* overexpression line (TICox); the analysis of circadian period result for TICox showed that TICox lines displayed similar short period phenotype as *tic*, and the similar phenotypes of leaf morphology were also observed. As *tic* mutants displayed drought tolerance, thus, to learn more about TIC in diverse physiological processes on drought stress response pathway, I investigated the phenotype of drought tolerance of *tic* mutant in Ws (Wassileskija) background and analysis the expression of key circadian gene *TOC1* which also involved drought

response in ABA depended pathway and downstream genes in *tic* mutants. , *tic* mutants in Ws background displayed similar drought tolerance phenotype and the expression condition of ABA-related gene (*ABAR*) compared with *tic*. Next, the *tic* retro-complementation line of TIC with mutagenesis of No.351 serine to alanine displayed higher drought tolerance compared to *tic* retro-complementation line of TIC and wild type.

Next, to investigate the TIC biochemical function and whether TIC can serve as substrates for AKIN10 which could impact energy metabolism for clock-oscillator function of AKIN10, the TIC fragment 427 to 578 was germinated and the phosphorylation which was activated by AKIN10 was observed in kinase assay in *vitro*. Thus, I use the MS analysis to test the phosphorylation on TIC for AKIN10, the result showed that No.466 serine is the phosphorylation set in *vitro*. Furthermore, the phosphorylated No.466 serine on TIC was also detected in MS analysis of nucleoprotein in *vivo*.

In summary, these observations of phenotype and the biochemical analysis result indicate that TIC is in a protein complex, which in the regulation process could inhibit TIC overexpression. Furthermore, the phosphorylation-dephosphorylation modification could be a main regulation method of TIC. Therefore, TIC is a connector between drought stress response and the circadian clock, and also strongly supports that TIC plays a role in the input pathway of circadian clock caused by phosphorylation with AKIN10.

Keywords: Time for coffee, Circadian clock, Drought stress, Kinase, Phosphrylation

Content

1 Introduction

1.1 Chronobiology and circadian clock

1.1.1 Introduction of Chronobiology

In the long course of life's development and the evolution process, which is dominated by natural selection, many species evolved a biological rhythm. It can be regarded as one of the basic features of life. Biological rhythm research has become an important subject of modern natural science, which is named chronobiology. Biological rhythm is a periodic change of biological life activity. Period is a kind of rule of material movement; thus, life also has its own period. The period of life activity and every stage of this period were presented in every moment of life time. Biological rhythm was by the biological characteristics changes in different segmental regulation over time and exists in the different levels of life, including the ecological system, community, population, individual, organ, tissue, cell, biological macromolecule; all these have their own rhythm.

In short, chronobiology is a science used to research the biological rhythm phenomenon. Biological rhythm is used to explore the mysteries of life and scientific discovery process; it has revealed a very important class of biological phenomenon.

The reason of the existence of circadian rhythmic, usually considered being life's adaption from the cyclical changes in nature (such as day and night, light and temperature changes in the phenomenon generated by the rotation of the year seasons and tides fluctuations, metabolisms) in the long evolutionary history.

1. Ultradian rhythm (ultradian, ultra-super, dian-day), a recurrent period or cycle repeated throughout a 24-hour day (shorter than 24 hours). For example, the descriptive term ultradian is used in sleep research in reference to the 90–120 minute cycling of the sleep stages during human sleep.
2. Circadian rhythm (circadian, circa-around, dian-day), external rotation of the earth is mainly thought to be due to the changes caused by the length of day (photoperiod, photoperiodism) and diurnal temperature variations (temperature cycles), circadian rhythm common manifestations of vibration angle change

leaves of plants, petals and pores' opening and closing, the human body temperature and hormone levels change nearly biorhythm cycles for 24 h.

3. Tidal rhythms (circatidal), external moon's gravitational effect on the Earth's surface and the formation of ocean's cyclical fluctuations, many marine organisms exhibit corresponding tidal rhythm.
4. Month's rhythm (circalunal), the rhythm because of the moon's rotation around the Earth externally, such as female human's menstrual cycle.
5. Seasonal rhythms and years rhythm (circaseasonal and circannual), external factors thought to be due to the earth revolution around the sun, seasonal leaves and mature trees growing plants-rings, seasonal breeding animals, sleep, and seasonal migration of birds belong to this category. Additionally, some of research in recent years report that cycle and rhythm phenomenon may relate to solar activity cycle (circadecennian).

Before Chronobiology came into scientific research areas, people had already started to observe nature and organisms to be rhythmic, and further learned and guided their practice. For example, in ancient China, as early as in the "Book of Changes" has been described in the sentence "The changing of heaven and earth builds the seasons," this can be seen to some extent on the nature of the ancestors of the Chinese national rhythmic infancy cognitive. Thus, the research of biological rhythm became an important part of modern biological science research and has critical guidelines for other biology research areas, otherwise, the consciousness of biological rhythm was already existed in acknowledgment of human civilization for a long time.

1.1.2 The Circadian clock

The most in-depth and extensive research of the regulation of biological rhythm cycle as nearly 24 h cycle rhythm phenomenon; termed the circadian clock. The word "circadian" is originally from Latin word "circa (around)" and "dias (day, 24 h)." It means that the rhythm cycle is nearly 24 hours. The clock experiments of modern

natural science were starting in 1720. Jean-Jacques d'Ortous de Mairan observed that the mimosas' leaves kept the same rhythmic opening and closing phenomenon under constant dark conditions as nature light conditions.

Study of the circadian clock in the biological circadian rhythm phenotype by mathematical analysis and calculation methods, common basic parameters include period, amplitude, phase and a median or mean. Period refers to a change in the cycle of the oscillation curve elapsed time. Amplitude refers to the change in the magnitude of physiological variables, when the cosine function to describe the circadian rhythm, the amplitude is half the difference between the maximum and minimum. Phase in chronobiology is the time point to physiological characteristic values of variables appearing in circadian rhythm changes in the mathematical formulation, the general common peaks or troughs phase study. Median (middle value) averages biorhythm related physiological variables studied data (Figure 1.1.2-1).

Subsequent research showed that the circadian clock phenomenon is quite widespread; the main mode species of circadian clock study include *Cyanobacteria, Chlamydomonas reinhardtii, Neurospora crassa, Caenorhabditis elegans, Drosophila melanogaster, Danaus plexippus, Arabidopsis thaliana, Mus musculus* and *Homo sapiens*, it covered most of the mode species for biology research.

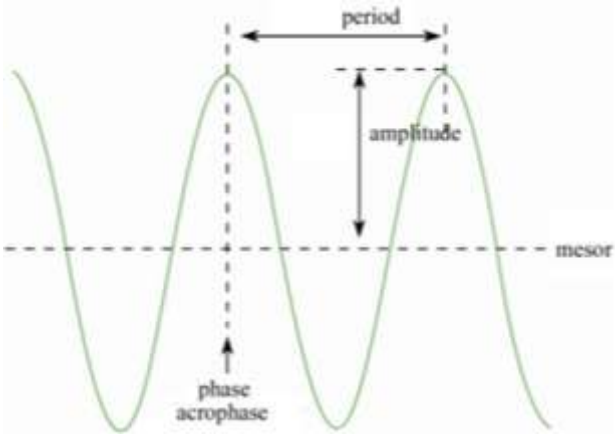


Figure 1.1.2-1 Explain of phase acrophase, mesor, amplitude, period. The phase acrophase is the lengthwise middle line of the waveform, the mesor is the horizontal middle line of the waveform, the period is distance between beside peaks, the amplitude is the height from mesor to pick, the period is distance between beside peaks.

In the beginning, study of the biological clock has experienced slow growth, in 1950s'; the main features regulation of 24 h circadian rhythm had been established. It includes:

1. Circadian clock is an endogenous circadian regulation It is independent of external environment periodic influence such as light condition and temperature. It is continued to 24h of periodic freedom self-sustaining. The circadian clock is a genetic result, it is controlled by the cell, and can predict cyclical changes in time and the environment.
2. Regulation of circadian clock can be entrainable by signals (such as light, temperature, etc.) from environmental surroundings, and further can be reset. As an example, when people travel to different time zones, they correct their watch. The circadian clock can be regulated to adopting the environment better.
3. The circadian clock regulation has the temperature compensation phenomenon. The circadian clock can keep the rhythm stable in a small temperature changing area.

1.2 Plant circadian clock

1.2.1 Introduction of plant circadian clock

Study of plant circadian clock has a long history. Advances in biotechnology have expanded the depth and breadth of research. The circadian clock regulates almost all of the plant participates of metabolism, growth and development, and causes the plant to have a 24h circadian rhythm which is synchronous with the external environment of light and temperature [1]. The circadian clock regulation mechanism ensures plants benefit in response to environment from transcription level to physiological and biochemical level. This nearly 24h rhythmic oscillation can decrease unnecessary consuming of energy and organics, and increase the productivity competitive and viability [1-4].

Normally, the plant circadian clock function has been considered to be uncoupled and un-tissue specificity, but a recent study performed a comprehensive tissue-specific analysis of leaf tissues, and discovered that the vasculature and mesophyll clocks unbalance regulate each other in Arabidopsis. The plant circadian clock in the vasculature was obviously different compared with other tissues without environmental influence, and affects circadian clock regulation in other tissues [5].

Unlike the plant circadian clock, the mammalian circadian clock is made up of many different parts, and showed strong tissue specificity. They maintain the physiology cycle from different tissues and organs of the body. The main circadian clock is located in the suprachiasmatic nucleus (SCN); it is a pacemaker of circadian rhythm. A certain number of collective behaviors of circadian rhythms were controlled by clock, such as movement, sleep, body temperature and endocrine. SCN has inner genetic rhythmic period, and it is affected by the light signals in the environment and some chemicals.

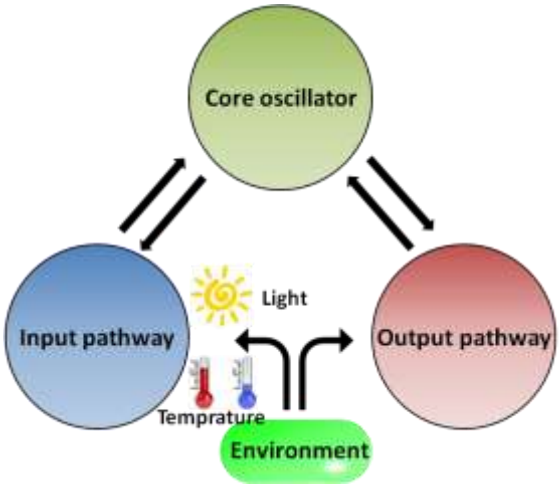


Figure 1.2.2-1 Plant circadian clock system composition, the crosstalk between the core oscillator, environmental inputs pathway and outputs pathway. The clock is regulated by light/dark and warm/cold cycles. Otherwise, the clock also controls multiple output pathways, some of which feedback to the clock as input signals. Both inputs and some outputs are gated by the clock.

1.2.2 Input pathway of plant circadian clock

The plant circadian clock system consists of three parts: input pathway, core oscillator, and output pathway. However, these three parts are not three independent

existence divisions. The signaling networks between them cross together; each consisting of an organic whole response to the regulation of plant growth and development and adaptation to the environment (Figure 1.2.2-1).

The current knowledge has demonstrated that the perception of the Arabidopsis circadian clock input pathway is mainly through circadian-related receptors. Phytochrome signaling pathways and their associated light components feel external light signals, and the regulation of circadian clock gene expression core loop to re-set the clock.

Temperature compensation is an important feature of circadian rhythms, but the research of plant circadian temperature compensation mechanism is not very in-depth. The CCA1, CK2, PRR7, PRR9 and GI gene more positively play an important role in the temperature compensation mechanism [6-10].

In recent studies, TIC was also found to be an important regulator in the input pathway of the circadian clock with Arabidopsis circadian mutant *tic*-1. In this study, the *tic* mutant showed a range of clock phenotypes, which was included by free-running circadian rhythms under light and darkness [11]. This mutant was also altered in rhythmic gating of light activated gene expression. The current knowledge of the Arabidopsis circadian model is that CCA1 and LHY repress the expression of evening-acting genes. Since both CCA1 and LHY expression were changed in the tic mutant, the expression of evening clock genes might be indirectly affected through a feedback oscillation. Therefore, in previously investigations of *tic*, the expression profile and waveform of the evening clock genes GI, ELF3, and ELF4. Next, the GI:LUC, ELF3:LUC, and ELF4:LUC fusion genes were introduced into the *tic*-1 mutant through crosses, and clock responses of transcription were monitored in these lines. ELF3 expression in *tic* was highly dampened both under constant light conditions and in constant darkness. By contrast, both GI and ELF4 expression maintained a low level of rhythmicity in *tic*, although both genes displayed reduced

amplitude compared with the wild type. As well, these two genes displayed a short-period response [11-12].

1.2.3 Core oscillator of plant circadian clock

The second part is clock core oscillator. Basically, the model plant Arabidopsis' core oscillator of clock system can be described of the complex regulatory networks into three loops [13] (Figure 1.2.3-1).

1.2.3.1 Core loop

The core loop was the first to identify three important core genes CCA1/LHY and TOC1 as the main feedback path. Where in the CCA1/LHY, one pair MYB similar transcription factor to inhibit the expression of TOC1. Thus TOC1 directly or indirectly, by expression of an unknown component regulatory CCA1/LHY, thereby constituting a complete regulatory processes. The new study found that members of the CHE in TCP transcription factors family, which also play an important role, CCA1/LHY can inhibit the expression of CHE, and CHE protein accumulation in turn will reduce the expression level of CCA1, while TOC1 can be combined with CHE and antagonistic it [14].

It is worth noting that the new findings overturned previous TOC1 promoting transcriptional activation of inference. It proved that TOC1 can be used directly as DNA binding transcription factor, combined with the CCA1/LHY's promoter on the sub-specific components, inhibit the expression, though it is a opposite conclusion with the previous clock model, but also gives some experiments to verify the need for further interpretation and inference [15].

1.2.3.2 Morning loop

The second loop is the morning loop, by CCA1/LHY and PRR7/PRR9, of which CCA1/LHY in the subjective day morning reached peak expression. It promotes transcription of PRR7/PRR9, while PRR7/PRR9 in turn suppresses CCA1/LHY expression. In addition, two other key clock genes LUX and ELF3 can also directly inhibit the expression of PRR9 [16-18].

1.2.3.3 Evening loop

The third cycle is the evening loop, the main component of TOC1 and unidentified Y (GI may be partially exercised the function of Y), TOC1 suppress the expression of GI, and GI promotes TOC1 expression, while GI's transcription is inhibited by CCA1/LHY [13]. In addition, PRRs (pseudo-response regulators) in Arabidopsis circadian regulation is essential. There are total of five Arabidopsis genome PRRs (TOC1/PRR1, PRR3, PRR5, PRR7, PRR9). The TOC1 is the first clock gene identified from short period mutants [19].

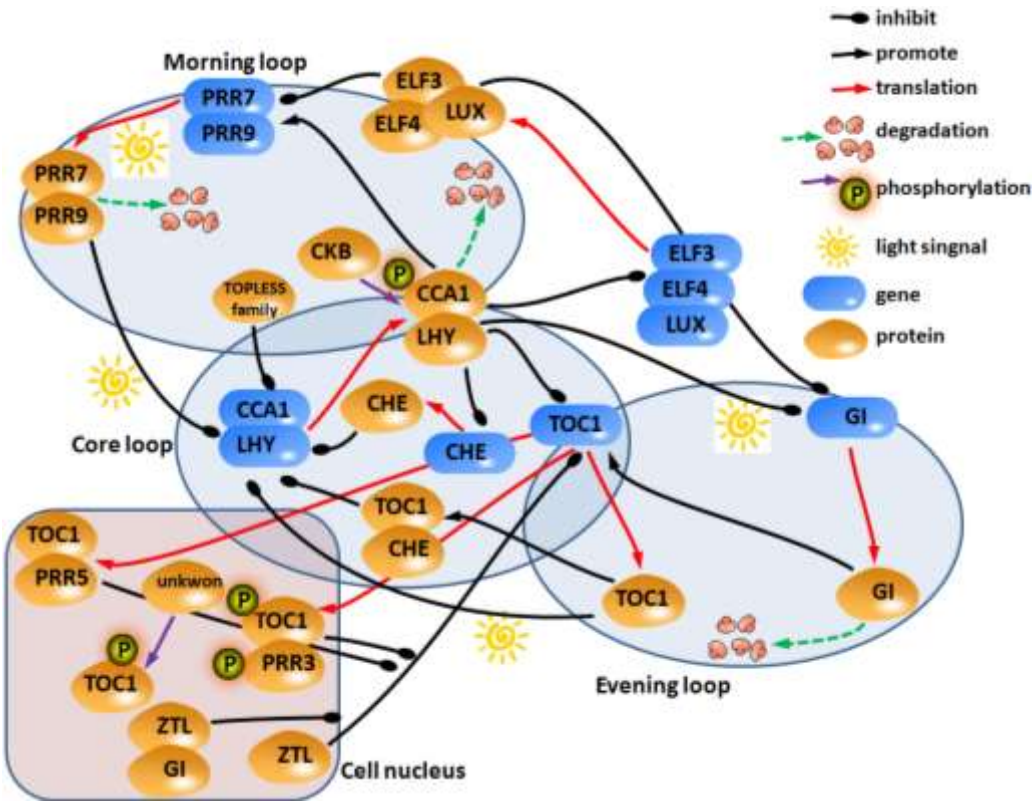


Figure 1.2.3-1 The main single pathway of plant circadian clock. CCA1, LHY, TOC1 compose the core loop, CHE play mediation role in this negative feedback loop, CCA1, LHY activated PRR7, PRR9 and PRR7, PRR9 inhibited CCA1, LHY, thus, they compose the morning loop, the TOC1 is inhibited by GI and GI activated TOC1, this regulation compose the evening loop.

Several publications have revealed that TOC1 belongs to one of five PRRs genes' family. Studies show a more complex regulatory expression of PRRs, which have a transcriptional regulation and post-transcriptional regulation. PRRs

transcriptional level of the most interesting phenomenon is that five genes regulated not only by the circadian clock, and their oscillations as concert quintet, their level of transcription peaks (peaks) in turn appears in intervals of about 2 h: The early morning peak expression of PRR9 before a pair of important clock genes CCA1/ LHY after about expression reached a peak in the morning (early in the morning); followed PRR7, PRR5, PRR3; TOC1 expression peak appeared in the early evening[20].

PRRs post transcriptional regulation is more complex, PRMT5 affects the alternative splicing of the [21] PRR9's mRNA, while phosphorylation events exist on PRRs at the protein level, other studies have revealed the phosphorylation determines the stability of PRRs, and the fate of their participation in the protein complex (stability and subcellular localization) [22-24]. The latest results also demonstrated PRR5, PRR7, and PRR9 by interacting with TOPLESS/TOPLESS- RELATED protein family, thereby inhibiting the expression of CCA1 and LHY, thereby regulating circadian clock [25].

At present, post-transcriptional regulation of the circadian clock studies have been focused on the study of SKIP genes, and confirmed the alternative changes of the mRNA splicing patterns can affect many circadian clocks' key genes' splicing, then play a role regulation of the circadian clock [26].

1.2.4 Output pathway of plant circadian clock

The adaptive part of the plant and its advance adjusted from the circadian clock system which has the "predict" ability of the environmental changes in the future, such as photosynthesis-related genes and proteins' transcription and translation in advance, to reach the maximum photosynthesis efficiency. Stomatals' opening and closing as well as periodic rhythmic and expression of stress resistance genes may aid plants in minimizing wastage of energy and resources, and cause resistance to biological and abiotic stress. This proved that if the plant circadian clock has synchronization with the Earth's rotation, then it will give the advantage of plant

growth and development and adaptability [3]. Moreover, the clock explains the phenomenon of heterosis from improves the plant's environmental adaptability effects [2] (Figure 1.2.3-2).

1.2.4.1 Biological stress response

The experiments of Arabidopsis against downy mildew showed that the CCA1 downstream genes may be involved in some basic defensive reaction and causes the plant to expect the time of pathogen infection, targeted to mobilize the appropriate defense genes expression [27]. The plant circadian clock has also been shown to advance "predict" feeding behavior of insects, through the regulation of jasmonic acid-mediated defense responses with suitable to fight against insects [28]. Another member of the core clock cycle system TOC1 (PRR1) was first cloned as ABI3 interacting protein (ABI3-interacting protein 1, AIP1) in seeds [29].

1.2.4.2 Abiotic stress response

TOC1 been shown to directly combine ABAR promoter and regulates the expression of ABAR periodically, while the ABA can up regulate TOC1. Therefore TOC1 is considered to play as a molecular switch between drought stress signaling pathways and circadian clock.

Studies have shown that at the Arabidopsis TOC1's same family genes PRR5/PRR7/PRR9 in plant adaptation to low temperature, high salt and drought stress also plays an important role, the *prr5*/*prr7*/*prr9* triple mutant plants' ABA content is significantly improved compared to the wild type, and the microarray result also showed that critical ABA biosynthesis genes are significantly upregulated [30-31].

1.2.4.3 Stomatal opening

Stomatal movement regulates leaves' CO2 exchange and moisture loss, while the opening and closing of stomata is also under circadian regulation. Recent research shows that in drought stress conditions, ABA effects on stomatal in the morning is significantly higher than in the afternoon, the *toc1* mutant has weaker ABA-mediated resistance in response to drought. Ca^{2+} as one of the most important second messenger

in plant cells, not only plays an important role in the growth and development of plants, but also involves many adversities signal transduction process [32-35].

1.2.4.4 Calcium ion concentration

Johnson et al. [36] in 1995 found that the presence of intracellular free calcium concentration near 24h rhythmic oscillation (circadian calcium oscillation) [37-38]. Xu et al. confirmed that red and blue lights are the regulation of cytoplasmic [Ca^{2+}] with nearly 24h rhythm oscillations, Red-ray pathway mediated mainly by PHYB, Blu-ray through CRY1, CRY2 double regulation. Blu-ray can improve the cytoplasm [Ca^{2+}] level; this regulation requires the joint participation of PHYB, CRY1, CRY2 to complete.

ELF3 (EARLYFLOWERING3) is like a gating, directly through the control of the circadian clock to adjust the light input pathways of intracellular calcium ions rhythm. The study also showed that the core Arabidopsis circadian regulation gene CCA1 and LHY participate in [Ca^{2+}] and CAB2 (CHLOROPHYLL A/B BINDING PROTEIN2) rhythm control, but in TOC1 different mutants *toc1*-1, *toc1*-2, [Ca^{2+}] cyt and CAB2::LUC rhythm has significant differences. This difference shows the existence of different output pathways, calcium rhythm and CAB2::LUC plant circadian rhythms may be separate from the core mechanism of different negative feedback loop to regulate [38]. At present, the question of where is the calcium ions which work on calcium rhythm oscillations from (extracellular or organelle). There are some preliminary findings in cADPR pathway and CAS-IP3 pathway, but the state is still in debate [39-42].

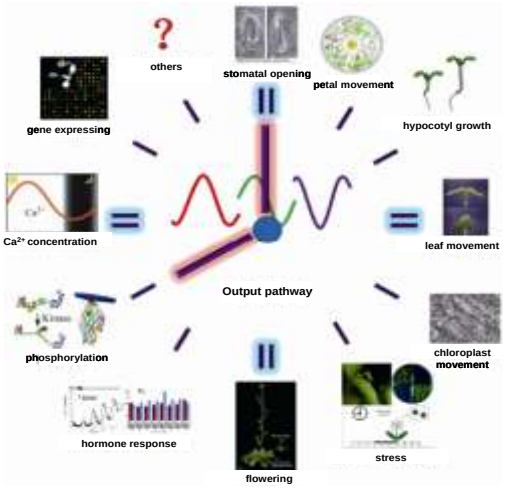


Figure 1.2.3-2 Plant circadian clock output pathway diagram

1.3 Abiotic stresses and plant circadian clock

Abiotic stress factors acting on the plants, the plants will cause a series of physiological and metabolic reactions, manifested as reversible inhibition of growth and metabolic seriously and even cause irreversible damage, leading to death of the whole plant. Various stresses, such as cold, drought, salinity are important factors in plant growth, yield and quality of crops. Therefore, the study of plant cold, drought and salt tolerance has always been a hot research field of botany. In recently years, with the rapid development of the deepening of the molecular mechanism of plant stress resistance and molecular biology techniques, resilience research has moved from physiological levels down to the molecular level, and promotes the development of genetic engineering of plants to environmental stresses (Figure 1.3-1).

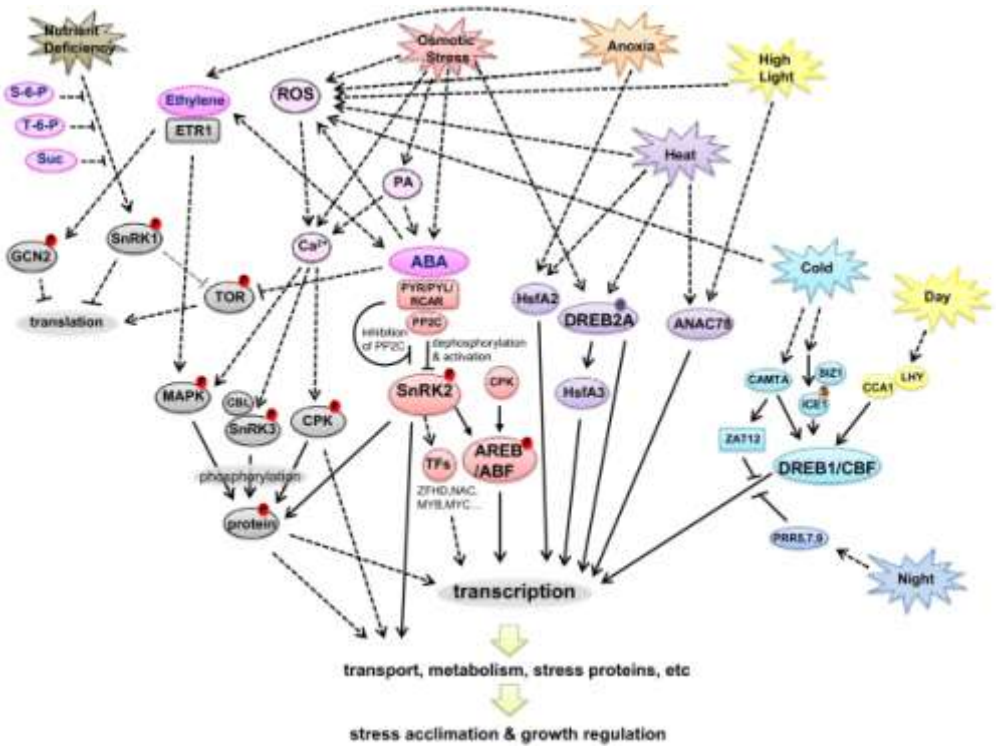


Figure 1.3-1 Signal pathway of different abiotic stress response in plants

1.3.1 The stress response function of Abscisic acid and connection with circadian clock

Recently, the collection of microarray data at different times of day shows that the ABA treatment data sets and cyclic adenosine diphosphate ribose (cADPR) [42] data sets have significant overlap. ABA response and clock genes overlapping are interesting, because a lot of stress responses pathways are controlled by ABA, such as drought stress, cold stress, and others.

Abscisic acid (ABA) is a 15-carbon isoprene as a basic unit of the type sesquiterpene plant hormones. It exists in various organs and tissues of higher plants, especially in mature and aging tissues or organs into dormancy. The higher plant, ABA is synthesized by carotenoid pathway. Early reactions, carotene precursors become carotene in the plasmid; carotene is a precursor of intermediate reaction. The mid-reaction, zeaxanthin become zeaxanthin aldehyde by 9-cis-epoxy carotenoid dioxygenase (NCED); the late reaction, zeaxanthin aldehyde through the short-chain

alcohol dehydrogenase (ABA2), aldehyde oxidase (AAO3) catalyzes the formation of ABA in the cytosol. ABA's most significant role in the regulation of plant development is the growth and plant responses to abiotic stress responses, such as seed germination, vegetative growth, bud dormancy, stomatal movement, mature embryo, leaf senescence, salt stress, low temperature stress, osmotic stress [43-45].

There are two stomatal regulating transduction pathways of ABA signal: ABA can promote stomatal closure and inhibit stomatal opening [46], although both lead to stomatal closure, but they are not easy to reverse the process. Due to drought stress, leaf water potential decline, increasing the chloroplast membrane permeability of the ABA, ABA-induced stomatal closure. After storing ABA is decreased in chloroplasts, ABA will re-synthesize. Recent research found that ABA content increased at least 20 times caused by water loss stress, causing the stomata to close. The ABA effect is due to its role in the cell plasma membrane free space outside the defense work. ABA reduces energy ATP- proton pump, cutting off H^+ and K^+ exchange channel, so that the water leakage turgor decreased stomatal closure. Once the water potential to restore normal chloroplast stops releasing ABA, ABA synthesis rate is significantly decreased [47].

In the circadian clock companion, the core oscillator TOC1 expression is induced by ABA, from what's publication [48] in response to and the core oscillator TOC1 not only depends on ABA and plant drought stress related, but also through the circadian and diurnal regulation of the H subunit of the magnesium-protoporphyrin IX chelatase stomatal regulation. Related, this pathway is also known as the genome uncoupled 5 control stomatal aperture (ABAR / GUN5). Overexpression TOC1 can hamper the stomatal closure lead to sensitive plants to drought stress [48]. Therefore, the clock and the associated stress reaction in response to ABA may signal through this connection, and increase endurance.

Many of the key genes involved in ABA synthesis and signaling have circadian expression model, causing overlapping results between ABA treatment microarray

and clock microarray. The stress response genes, which are induced during the daytime were induced, for example: EARLY RESPONSE TO DEHYDRATION 10 (ERD10) and 7 (ERD7), COLD REGULATED 15 B (COR15B) and A (COR15A) and A (COR15A) and RESPONSE TO DISSECATION (RD29A) [49].

Recent research shows that these genes are responsible for drought stress. Covington et al.'s results show that not only is ABA-induced gene expression in the daytime, but the key enzyme of ABA biosynthetic and ABA precursors are also controlled by the clock. The enzymes are involved in ABA biosynthesis, such as: the isoprenoid precursor's synthase CLOROPLASTOS ALTERADOS 1 (CLA1) and PHYTOENO SYNTHASE (PSY), the carotenoid synthase 9-CIS' EPOXYCAROTENOID DIOXYGENASE (NCED3) and ABA DEFICIENT 2 (ABA2) [49]. Recalling carotenoids lutein participation cycling in chloroplasts, in this way to avoid excessive absorption of solar energy, and through this loop control stomatal opening and closing [50], the clock seems to link heat from the sun during the day to drought stress, and in the night to give advance preparation.

In conclusion, because of ABA's biosynthesis and responsible genes have a way to link circadian clock, the stress and circadian clock genes share same expression model in some conditions. High numbers of salt and drought stress responsible genes are under circadian control [4]. Recently the genome tiling arrays result shows that it still has numbers of unannotated abiotic stress responsible regions in the genomic DNA. This means that stress responsible genes are a diversification. The data also shows that salt stress and osmotic stress basically share most genes compared to other stress treatment. The ABA treatment result is also similar as the aforementioned treatment, but the cold treatment and heat treatment shares the less results [51]. Dusk plays a role as an environmental signal for circadian control. At dusk, genes involved in starch remobilization and lipid modification reach their peak expression [52-53].

1.3.2 The regulation between circadian clock and DREB

In the dusk, the light to dark transition, environmental changes cause plant starch

remobilization and lipid modification going down regulation. This phenomenon gives plant an advance preparation of cold stress. The cold responsible genes C-REPEAT BINDING FACTOR 1/DEHYDRATION RESPONSIVE ELEMENT BINDING 1 (CBF1/DREB1) family of transcription factors also have circadian expression. For example the CBF1/2/3 are high light expression genes, but the targets of CBFs' transduction is delayed. As such, they can aid the plant to have more cold tolerance after the dusk [54].

Otherwise, a lot of CBF/DREB genes regulation is also resistance to salt and drought stress [55]. The CBF/DREB transcription factors are in the ABA independent single pathway, but they share the resistance profile with ABA dependent genes.

Franklin et al. (2007) results show that in the low R/FR ratio (increased far-red light) light condition, (They used a cover to shading the plant) the CBF1/2/3 all has higher expression compared to normal conditions. After experiencing a low R/FR treatment, the plant's cold tolerance is increased. In the dawn and dusk or longer in higher latitudes region, the stronger far-red light has happened. In the dusk, the circadian clock gives the plant advance preparation of cold stress, the CBF expression's requirement of a low R/FR seems a gating from cold stress responses to the circadian clock [56].

In conclusion, ABA and light signals are important linkers between the cold response and circadian clock, making transcriptional interconnects in the input and output pathways of circadian clock.

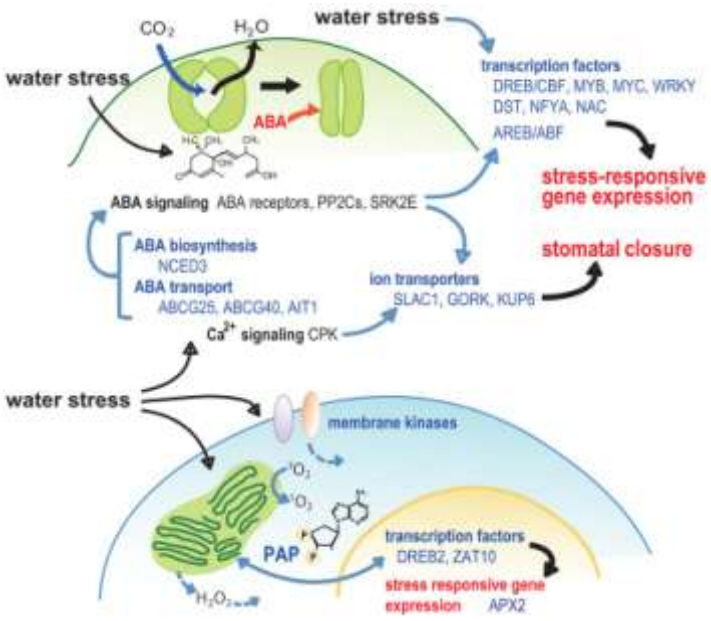


Figure 1.3.2-1 ABA functional response of water stress

1.3.3 The relationship between circadian metabolism and stress

Dodd et al. (2007) showed that the molecular-genetic evidence mediating is the key piece between the plant circadian clock and metabolism. Their research also demonstrated that cyclic adenosine diphosphate ribose (cADPR) is synthesized from NAD by the ADP ribosylcyclase, peaks in the early morning and affects the oscillator. In the concentration of cADPR lengthened, the period of clock-controlled genes are decreased. The ADP ribosyl cyclase can be inhibited by nicotinamide, and the circadian calcium oscillations will be weak [42].

Recent research showed that 30% of all ABA responsive genes were expressed in a similar pattern than those from cADPR. As such, ADPR cyclase activity was induced by ABA [57]. Recalling that circadian clock microarray datasets overlapped with ABA transcriptomic profiles [49]. They suggested that with the thronging of cADPR, the circadian clock can link metabolism system.

The status of carbon availability throughout the day seems an important factor between the clock, ABA and energy. Previous research has set up several concepts to descript the mutants' name which involve sugar responses, such as glucose insensitive

(*gin*), sugar insensitive (*sis*) and sucrose uncoupled (*sun*). In these mutants, ABA response mutant phenotypes have also been found; some were allelic to ABA INSENSITIVE 4 (ABI4) and ABA DEFICIENT 2 (ABA2) [58]. These results showed that carbon availability is directly linked to ABA signaling.

As previously reviewed, the clock has a tight crosstalk with ABA gene expression profiles, and clock responsive genes' expression is influenced by carbohydrates; this is a very interest finding. Blasing et al. (2005) also showed that half of the circadian-controlled genes could respond to sugar. In conclusion, the cellular sugar levels seem to be a major contribution in the establishment of diurnal gene expression patterns [59].

In these studies, they describe the phenomenon that low sugar conditions can induce sugar-controlled gene expression, but high sugar can't. In the impaired starch synthesis of phospho-glucomutase (*pgm*) mutant, their sugar responsive genes were rapidly induced when the endogenous sugars levels diminished. During the light period, high sugar levels were present and gene expression did not change. However, in the wild type, transcriptional reprogramming occurred to declining levels of sugars at the end of the night [59]. The carbon availability changing in the daytime and circadian clock are all involved with photosynthesis. They are responsible for many cyclic patterns genes' expression in the natural day length.

Recent research also showed that the plants' starch is degraded in the night time and the consumption rate of starch reached the lowest peak in the end of dark time. This phenomenon indicated that the plant's carbon resources consumption also follows the time [53]. Therefore, the photosynthesis made up some carbon sources at dawn. Otherwise the previous research points out that there is a short-period mutant consumed its starch before the ending of night. This caused the expression of starvation response genes and also diminished growth.

In conclusion the light-dark cycles can make an advantage adjusting for carbon supplies, this preceding not through affecting with photosynthesis [53]. Dodd et al.

(2007) suggested that this phenomenon could cause the long period and/or starch accumulation, then that will be a clock mutant [42].

1.3.4 The clock mutants' stress phenotype

1.3.4.1 *toc1*

In the research of Arabidopsis mutant *toc1*, the results displayed altered plant responses to drought by controlling stomata aperture (see above and 1.2.5.2) and thus gas exchange. TOC1 became a promoter former binder of ABAR/GUN5. This demonstrated that the TOC1 effects on ABA responses, and can effect stomata opening [48]. Kant et al. (2008) used a functional genomic pipeline to search for genes involved in multiple abiotic stresses. In these previous studies, the mutants of two components of the core loop *cca1* and *lhy* are sensitive to salt, osmotic and heat stress [60].

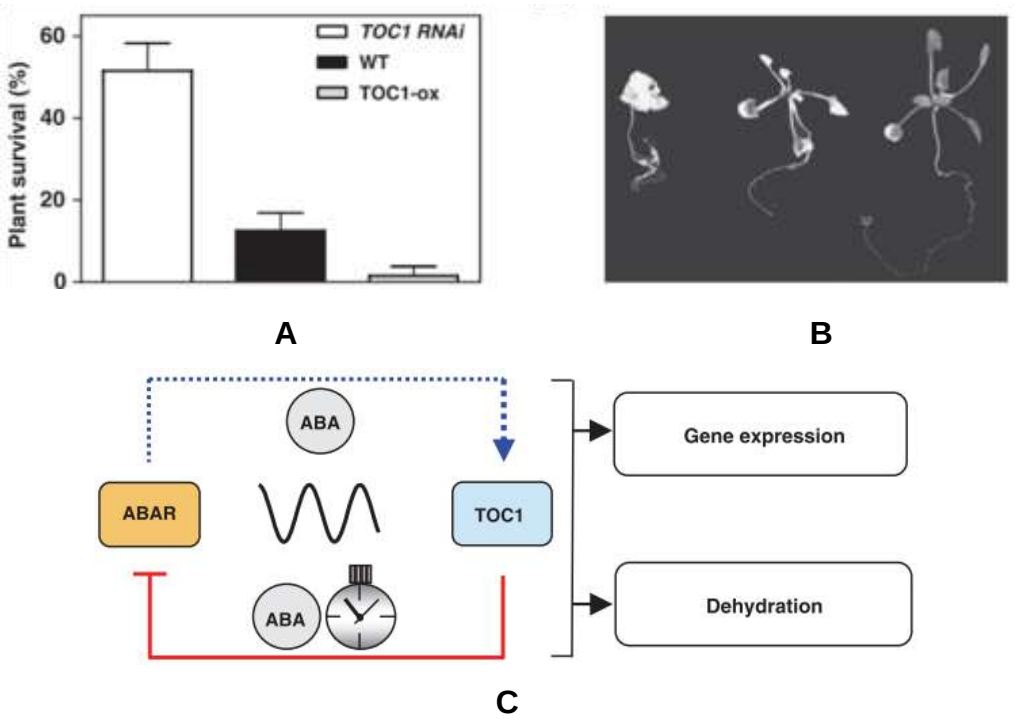


Figure 1.3.4-1 Altered responses to drought conditions of plants mis-expressing TOC1 (TOC1-1) (adapted from Legnaioli et al [48]).

A. Plant survival to dehydration stress on agar plates. Data are means±s.e.m. of duplicate experiments with at least 25 plants per genotype.

B. Representative photographs of TOC1-ox (left), WT (middle) and TOC1 RNAi (right) plants of the dehydration experiments.

C. Schematic representation depicting the reciprocal regulation between TOC1 and ABAR and the

implication of ABA and the circadian clock in this regulation.

1.3.4.2 *prr9/prr7/prr5*

In similar work to *toc1*, the triple mutant *prr9/prr7/prr5* displayed metabolic defects, but compared with the CCA1 overexpression line, they were different in tricarboxilic acid cycle (TCA). The mutant combination also displayed defects in biosynthethic pathways involved in chlorophyll, carotenoid, tocopherol and ABA. This triple mutant is sensitive to drought stress, but has a higher tolerance to freezing stress and upregulates cold responsive genes [30]. The more analysis of the *prr9*/*prr7*/*prr5* triple mutant gave the evidence for molecular link between metabolism and the circadian clock, and this is the first time in plants [61].

1.3.4.3 *gi*

The clock mutant *gi* showed lower increase in H_2O_2 and superoxide production compared to wild type, as well as reduced lipid peroxidation when oxidative stress was created by methylviologen application. The mechanism that leads to starch accumulation and oxidative stress resistance in *gi* is still unknown [62-63].

1.3.4.4 *tic*

In addition to the core oscillator, there are also some important companions have function for circadian clock, and some of them also involve stress response. Anthony Hall et al. (2003) characterize the *time for coffee* (*tic*) mutant that disrupts circadian gating, photoperiodism, and multiple circadian rhythms, with differential effects among rhythms.

This research showed that TIC is distinct in physiological functions and genetic map position from other rhythm mutants and their homologous loci. The plants analysis of rhythm also point to chlorophyll a/b-binding protein gene expression rhythm requiring TIC function in the mid to late subjective night. This gene has the name “time for coffee” because in contrast to the function of EARLY-FLOWERING3 (ELF3) in the late day to early night, TIC’s function just like human activity may

require coffee. The *tic* mutants also lost some circadian genes' expression. This research identified TIC as a regulator of the clock gene circuit [11].

The following research investigated how TIC functions within the circadian system. The result showed that the phases of evening clock genes in *tic* were all advanced under light/dark cycles without major expression level defects. In this, tic mutants had a short period compared with wild type, and this result indicated that TIC has a closer relationship with the core oscillator LATE ELONGATED HYPOCOTYL (LHY) than with CIRCADIAN CLOCK ASSOCIATED1 (CCA1), as *tic* has a specific LHY expression level defect [12].

In an in-depth investigation of the *tic* mutant, current research explored the mechanism of influence of TIC in plant growth and development, as initiated by a microarray analysis. The lost TIC function mutant *tic* causes a major reprogramming of gene expression that predicts numerous developmental, metabolic, and stress-related phenotypes, and it exhibited hypersensitivity to oxidative stress and abscisic acid, and this was associated with a striking resistance to drought stress. These phenotypes were connected to an increase in total glutathione levels that correlated with a readjustment of amino acids and polyamine pools. This result demonstrated that TIC is a central element that integrates and coordinates developmental, metabolic, and environmental signals [64].

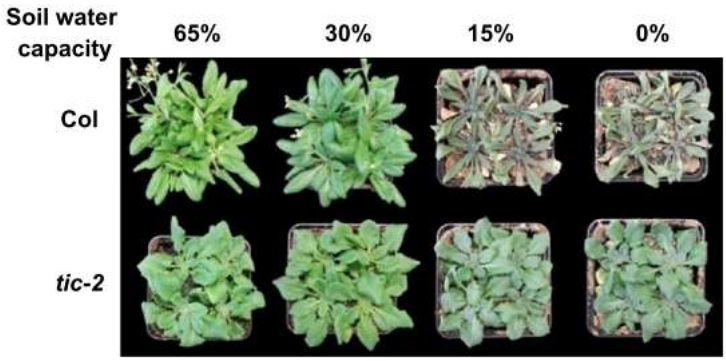


Figure 1.3.4-2 Photograph of Col and *tic*-2 under various levels of drought conditions (adapted from Sanchez-Villarreal et al. [64]).

1.3.5 Anthocyanins

The existence of anthocyanins either in part (flowers and fruits) or exclusively (leaves) in plants is an important reason of red, blue and purple coloration in various tissues of different plant species. Anthocyanins are water-soluble pigments derived from flavonoids via the shikimic acid pathway and appearing only in juvenile or senescing tissues, or they may be permanent [65]. As a class of flavonoids, anthocyanins do not only make flowers and fruits colorful, attracting animals for pollen dispersal and pollination, they also display a variety of biological activities. When anthocyanins became a medicinal treatment, it showed antiflammatory [66], anticarcinogenic [67] and antimicrobial properties [68]. Moreover, anthocyanins play an important role in plant stress responses. For example, in oxidative stress tolerance, it protects plants from ROS damage [69].

Further research shows the intraspecific responses of 12 winter wheat cultivars to different doses of ultraviolet-B (UV-B) radiation were analyzed and compared. It was found that the response indexes of plant anthocyanin were significantly correlated with the cumulative stress response indexes, indicating that anthocyanins are vital to UV-B tolerance [70]. Anthocyanins were also found to be a major contributor to the total antioxidant activity from blue wheat extracts, which are popular in food applications [71]. Low-temperature associated soluble carbohydrate accumulations are commonly associated with anthocyanin coloration, attenuation of growth and cold adaptation in cool-season grasses. Therefore, anthocyanin coloration was proposed as a useful phenotypic marker for soluble carbohydrate accumulation [72].

1.4 Introduction of Plant SnRK kinase family

Plant set protection mechanisms to deal with abiotic stresses in the development process of evolving, these stresses including drought, cold, high salt, high temperature and oxidation and others. Under these unfavorable factors, the plant can activate

related genes or alter the protein structure to protect a variety of metabolic reactions within the cell, thereby maintaining the integrity of the plant structure and function. Among them, the reversible phosphorylation of proteins is a major signal of higher plants subject to osmotic stress induced by one of the mechanisms. Through phosphorylation of proteins, cells can be adjusted in a range of life activities, such as cell mitosis, metabolism, intracellular signals, etc. to the growth and development of the organism and play an important role in regulating.

The plant SnRK (sucrose non-fermenting1-related protein kinase,) is a class of widespread plant protein kinase. It is a Ser/Thr protein kinase, participates in a variety of signal transductions in plants and plays a very important role in anti-stress physiology. According to conservative sequence similarity and activity of plant protein, the kinase domain of the family can be divided into three subfamilies: SnRKl, SnRK2 and SnRK3 [73-74], are widely involved in glucose metabolic pathways and other drought and salt stress the role of osmotic stress responsive. This review will focus on SnRK1 family's introduction.

1.4.1 Plant SnRK1 kinase family

The plant SnRKl, yeast SNF1, and mammalian AMPK in the structure and function have a more direct homology. The similarity of the amino acid sequence, SnRKl is divided into two groups; SnRK 1a and SnRK 1b [75] SnRKla was expressed in all plants, but SnRK 1b only exists in monocots plants, and the highest expression in seeds. The first to be cloned SnRKl cDNA sequence (cRKIN 1) derived from rye endosperm cDNA library, the amino acid sequence similarity with the SNF1 48% encoding a 57.7 kD protein [76].

Using Arabidopsis as an example, the Arabidopsis SnRK1 subfamily comprises SnRK1α1/SnRK1α2/SnRK1α3 (also named SnRK1.1/SnRK1.2/SnRK1.3, AKINα1/ AKINα2/AKINα3, KIN10/KIN11/KIN12, or AKIN10/AKIN11/AKIN12), the catalytic subunits of the SnRK1 complex. They are most related to SNF1 and AMPKα. In these subunits, only SnRK1α1 /SnRK1α2 can be expressed [77]. The Arabidopsis

SnRK1 subfamily also has regulation subunits such as the SnRK1β group, SnRK1βγ and SnRK1γ group. Each of these genes has since been renamed, and here I made a summary of these genes' names. (Table 1.5.1-1)

SnRKl plays a role in plants similar to that of yeast SNF1 and is involved in glucose metabolic pathways. It can be deduced that there is a similar plant sugar metabolic pathway in yeast and plants can be used instead of yeast SNF1 exercise SnRKl signaling function [76,78].

Table.1.5.1-1 Arabidopsis SnRK1 kinase family members' names

Name	Other name
SnRK1.1	SnRK1α1,AKIN10, KIN10, AKINα1
SnRK1.2	SnRK1α2, AKIN11, KIN11,AKINα2
SnRK1.3	SnRK1α3, AKIN12, KIN12,AKINα3
SnRK1β1	AKINβ1, KINβ1
SnRK1β2	AKINβ2, KINβ2
SnRK1β3	AKINβ3, KINβ3
SnRK1βγ	ATSNF4, AKINβγ, KINβγ
SnRK1γ1	AKINγ, KINγ, KING1
SnRK1γ2	-

1.4.2 The structure of SnRK1 kinase

The SNF1/AMPK/SnRK1 protein kinases are conserved throughout all eukaryotes and share a αβγ heterotrimeric structure (Fig 1) [79-81]. The catalytic subunit is named as α-subunit and has two parts; the activity domain [82] and the regulatory domain. There are 11 sub-domains and comprises the activation loop (also called T-loop) canonically fold displayed in the activity domain. Further, the regulatory domain has an auto-inhibitory sequence (AIS), which was shown to inhibit kinase activity [83-84]. However, this only happens in yeast and mammals.

In plants, the T-loop appears not to be inhibitory [85] and harbors an ubiquitin -associated (UBA) domain that was proposed to mediate the interaction with ubiquitinated proteins [86]. In addition, there is a KA1 (kinase-associated 1) domain on the kinases; this domain is responsible for the interaction with the regulatory subunits and the upstream phosphatases [87-89](Figure 1.5.2-1).

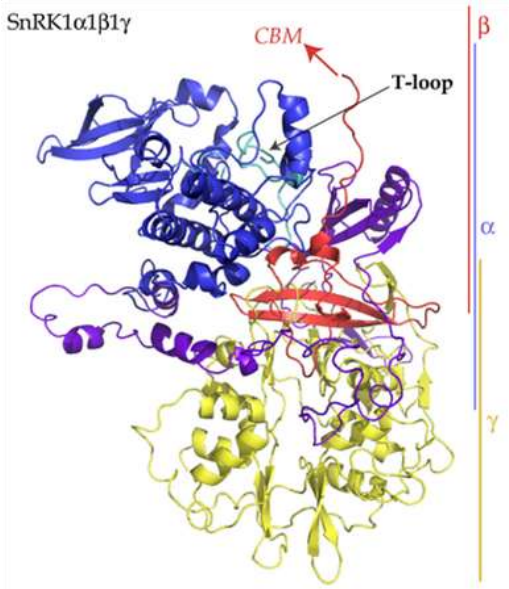


Figure 1.5.2-1 The structure of plant SnRK1 kinase (adapted from Croze et al [90]).

Plant SnRK1 also has two special subunits: the β3 subunit mentioned above and the βγ subunit a true γ-type subunit with an N-terminal extension containing a CBM [91].

As in Fig2, there are several heterotrimer compositions; they are possibly *in vivo* in all eukaryotes (up to 12 in Arabidopsis). This is likely the first level of regulation of these kinases, as for instance, different β-subunits within the SNF1 complex trigger differential localization [81].

1.4.3 The Phosphorylation of SnRK1 kinase

Phosphorylation of a conserved threonine in the T-loop of the catalytic subunit (SnRK1α1T175 /SnRK1α2T176; AMPKα2T172; Snf1T210) is necessary for SNF1/AMPK/SnRK1 activity [77,85,92-94] (Figure1.5.3-1). Many protein kinases use a similar regulation mode. The regulation mode is probably required for proper

alignment of ATP in order to allow its interaction with the catalytic lysine [95]. It is noted that the phosphorylation site of Arbidopsis SnRK1α1 (also named SnRK1.1 or AKIN10) was No. 175 Serine not Threonine.

In plants, analyses of total cell extracts reveal no differences in the phosphorylation levels of the activation loop between control and stress conditions [89,93]. This result may show that mechanisms of plant phosphorylation levels are not dependent on the controlling activity of stress. Recent research of SnRK1 phosphorylation following size fractionation revealed higher T-loop phosphorylation when the catalytic subunit was incorporated into a complex [96]. This result shows that the stress only induces the catalytic subunits' phosphorylation, which is incorporated into the complex. As such, the analyses of total cellular SnRK1 have missed this level of regulation.

1.4.4 The function of SnRK1 kinase

So far, the understanding of the physiological role of SnRKl is not as comprehensive as yeast and mammals such as SNF1, AMPK. However, the available data indicate that, SnRK1 to phosphorylate and activate 3-hydroxymethyl-3-glutaryl coenzyme A reductase, a phthalocyanine (HMGR) [97], sucrose phosphorylase.

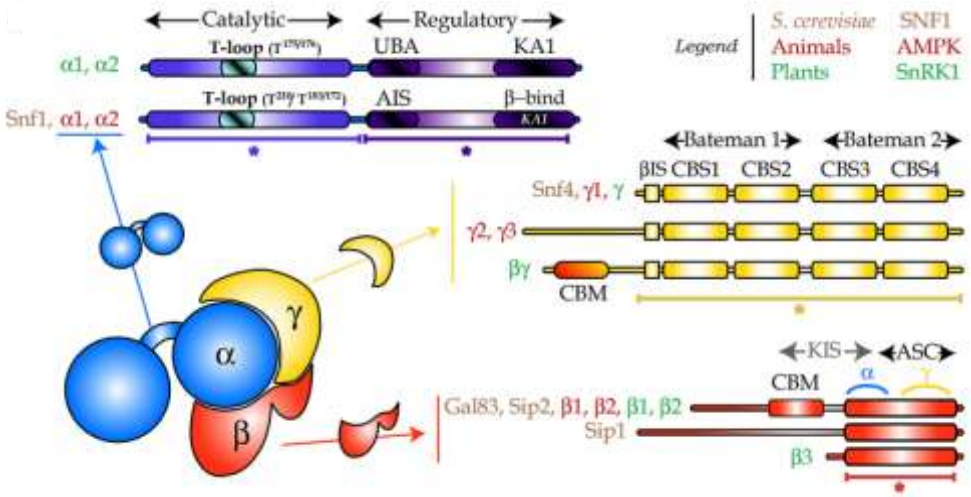


Figure1.5.3-1. The Phosphorylation site of SnRK1, SNF1, AMPK kinase family (adapted from Rodrigues et al [89]).

(SPS), nitrate reductase (NR) and trehalose-phosphate synthase (TPSS) 4 plant metabolic processes important enzymes [98].

In addition, SnRK1 also plays a role in regulation on the developing plants. Antisense expression traits SnRKl barley pollen abortion occurs, pollen abortion smaller pear-shaped, no starch or starch content rarely. Halford et al (2003) believes that the pollen sterility usually associated with the accumulation of starch and sucrose metabolism related. Under normal circumstances, SnRKl responds to high sugar/low glucose signals and induces the expression of related genes. In antisense expression SnRKl pollen, cannot convert enzyme expression corresponding to the use of exogenous sucrose, and so pollen abortion occurs [99].

1.4.4.1 Glucose metabolism

In plants, T6P is an inhibitor of SnRK1. T6P extractions show SnRK1 activity in Arabidopsis seedlings at concentrations in the micromolar range. This inhibition was also observed in extracts from other Arabidopsis tissue and other plants (spinach, broccoli, and cauliflower), with the exception of fully mature leaves [100]. Interestingly, no function of T6P was found in the activities from yeast, nematodes, flies, or human liver, this result shows that this is plant specific process (Figure1.5.4-1).

However, the inhibition can be restored by supplementing the supernatant from immunoprecipitated seedling extracts indicating that an intermediary factor separable from SnRK1 activity is necessary for inhibition of SnRK1[101]. During wheat grain development, SnRK1-induced and SnRK1-repressed marker gene expression changes in the different tissues of the seed are correlated with changes in T6P levels, further supporting SnRK1 inhibition by T6P [100]. A similar correlation was found in Arabidopsis seedling extracts. Except T6P, SnRK1 activity is also inhibited by other sugars, such as glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), glucose, and sucrose [96] (Figure1.5.4-1).

Supply of exogenous non-phosphorylated glucose and sucrose (5-50 mM) to seedlings and mature leaves also inhibits SnRK1 activity [77]. On the other hand,

several studies have reported an induction of SnRK1 activity by sucrose or SnRK1-dependent activation of gene expression or enzyme activity by sucrose [102]. Such effect may be due to the heterotrophic nature of the material employed, in which SnRK1 may be regulated differently than in autotrophic leaves, or to the high sugar concentrations used, which may trigger stress and defense responses [103]. Finally, inhibition of SnRK1 by ribose-5-phosphate and ribulose 5-phosphate [96] was also observed. SnRKl also has indirect regulation of carbohydrate metabolism, and adjusts synthesis of sucrose [104].

It is noted that in animal, the inactivation of the animal clock components Bmal1(Mop3) and Clock inhabit the accumulation day-night changing of glucose. Gluconeogenesis is disabled in clock-mutants *Bmal1* (Glu). However the relationship between the clock and glucose in the plant is still unclear.

1.4.4.2 The stress and ABA response

In the case of plants, ABA is an important factor of stress response. There is an increasing number of studies linking SnRK1 to the ABA phytohormone (Figure1.5.4-1). SnRK1 appears to play a central role in processes well known to be under ABA control, such as seed maturation and germination. Furthermore, Arabidopsis plants overexpressing SnRK1α1 are hypersensitive to ABA during germination and early seedling development, consistent with the phosphorylation by SnRK1α1 of FUSCA3, a central transcription factor regulating seed maturation [105].

Recent studies demonstrated that in mature photosynthetic tissues ABA activates SnRK1 through inhibition of its negative regulators, the 2C-type phosphatases ABI1 and PP2CA [104]. This may allow the complementation of the ABA response with a more general one triggered by SnRK1 and directed toward a metabolic and transcriptional reprograming. Additionally, the presence of ABA may potentiate SnRK1 signaling by blocking its inactivation and may allow SnRK1 activation in distant tissues not directly exposed to energy stress. Interestingly, ABA represses SnRK1 signaling via plant-specific SnRK1A-interacting negative regulators during

germination and early seedling growth and induces SnRK1 degradation in wheat roots [106]. This suggests the effect of ABA may differ between autotrophic and heterotrophic tissues in a similar manner as animal hormones control AMPK in opposite manner in different tissues (Figure1.5.4-1).

SnRKl also takes the response of plants to different stress. Lovas et al (2003) studies showed that antisense expression potato StubGAL83 (encoding potato SnRKlβ subunit) gene in transgenic plants, showing NaCl sensitive characteristics, while also severely inhibiting root growth. SnRK1 is also associated with plant disease resistance [107]. Hao et al (2003) studies showed antisense expression Arabidopsis SnRK1 in tobacco, displaying the characteristics of the virus-sensitive, while overexpression SnRKl the improved resistance to transgenic plants [108] (Figure1.5.4-1).

Full understanding of plant SnRK1 will also require the identification of further upstream regulatory components as well as a better characterization of their effects. In addition, the passive role of these upstream components, traditionally regarded as being constitutively active should be revisited, as an increasing body of evidence supports metabolic and hormonal regulation at least of the SNF1/AMPK/SnRK1 phosphatases. Identification and characterization of the upstream regulators may also be crucial for understanding the connection of these signaling cascades to other important pathways, as demonstrated for the dual role of ABI1/PP2CA phosphatases in SnRK1 and ABA signaling [89].

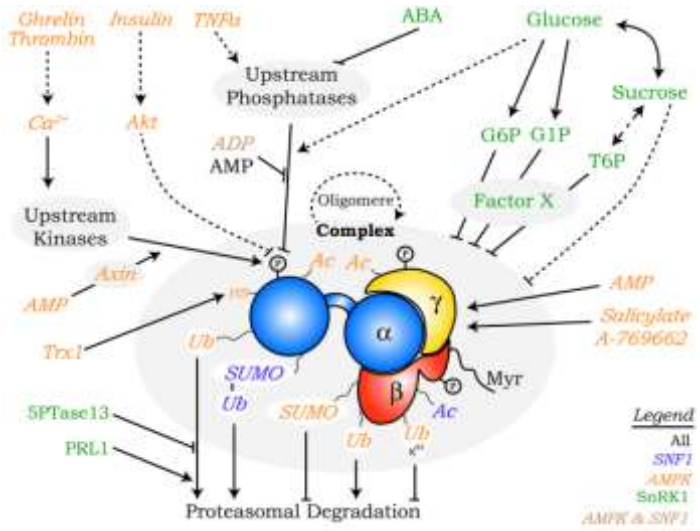


Figure1.5.4-1 The upstream and downstream regulation of SnRK1, SNF1, AMPK kinase family (adapted from Croze et al. [90]).

Aim of this thesis

The current understanding of the role of TIC in clock entrainment is still unclear. Combined with the result of TIC and AKIN10 interaction, this thesis aims to explain how TIC participates in entraining the clock through the input pathway and what is the role of TIC in entrainment and is AKIN10 upstream element regulates TIC in the input pathway of the circadian clock. Previously reported phenotypes of stress tolerance in *tic* mutants, especially drought stress phenotype, is also needed to explain if TIC an important connector between stress and the circadian clock. With those questions defined, the objectives of my thesis work were as followed:

1. Detect how TIC works as a connector between stress and the circadian clock in the input pathway. Particular objectives:

   1.1 Perform and analyze the *tic* mutants drought stress tolerance phenotype and the mechanism, in previously research of drought stress function of TOC1; detect the difference of the express model between wild type and *tic* mutants; figure out the TIC function in connection between stress and circadian clock.

   1.2 Characterize how TIC effects and responds to drought stress. My hypothesis is

that TIC responses to drought stress through phosphorylation diphosphorylation modification. I will use some web service to predict the potential phosphorylation sites, and design experiments to verify the function of potential phosphorylation sites.

2. Detect how TIC acts downstream of AKIN10 in protein interaction and phosphorylation to connect glucose metabolism, energy and circadian clock in the input pathway. Particular objectives:

   2.1 Detect the phosphorylation relationship between TIC and AKIN10, use the prokaryotic expression build the TIC protein fragment and verify whether it is AKIN10 kinase phosphorylation reaction substrates.

   2.2 Investigate and clarify the interaction between TIC and AKIN10, I plan to map phosphorylation sites on TIC for AKIN10, and design experiments to detect the function of the phosphorylation sites.

2 Material and methods

2.1 Materials

2.1.1 Mutant lines

Table 2.1 Mutant and transgenic lines previously made

Mutant and transgenic lines previously made	Background	Reference
tic-1	Ws-2	Anthony Hall, 2003
tic-2	Col	
AKIN10	Col	
TICox-2	Col	
TICox-15	Col	

Table 2.2 Luciferase lines

LUC marker	Background	Selection marker
TOC1:LUC	Ws-2	Hygromycin
tic-1-TOC1:LUC	Ws-2	PPT
GI:LUC	Col	Hygromycin
tic-2-GI:LUC	Col	PTT
AKIN10- GI:LUC	Col	PTT, Hygromycin
tic-1-TICS351A-TOC1:LUC	Ws-2	Hygromycin

2.1.2 Chemicals

1,4 Dithiothreitol (DTT) (biomol, #04010.5)

2-(N-morpholino)ethanesulfonic acid, MES (Duchefa, #M1503)

3′,5′-Dimethoxy-4′-hydroxyacetophenone, Acetosyringone (Sigma, # D134406)

Acrylamide (29:1) (Roth, #A124.1)

Adenine hemisulfate (Sigma, #A-9126)

Agarose (Bio-Budget, #10-35-1020)

Ammonium persulfate (APS) (Sigma, #A7460)

Bactoagar (BD, #214040)

Bacto-tryptone (BD, #211705)

Beef extract (BD, #212303)

Boric acid (Merck, #1.00165)

Bromophenol blue (Sigma, #47522)

Carbenicillin (Sigma, #C-1389)

Chloramphenicol (Sigma, #C-0378)

Chloroform (Merck, #1.02445)

cOmplete® EDTA-free tablets (Roche, #11873580001)

cOmplete® Mini EDTA-free tablets (Roche, #11836170001)

Dimethyl sulfoxide, DMSO (J.T. Baker, #7157)

DL-Phosphinothricin, PPT (Duchefa, #P0159)

D-Luciferin (Synchem, #S039)

Ethanol (J.T.Baker, #8006)

Ethidium bromide (Sigma; #46067)

Ethylenediaminetetraacetic acid, EDTA (Merck, #944)

β-estradiol (Sigma; #46067)

Gentamicin sulfate (Sigma, #G-3632)

Glutathione sepharose 4B (GE Healthcare Life Sciences, #17-0756-01)

Glycerol (Roth, #7530.1)

Glycine (Roth, #3908.2)

Hygromycin (Duchefa, #H0192)

Imidazole (Sigma, #56750)

Isopropyl β-D-1-thiogalactopyranoside (IPTG)(Roth, #2316.2)

Isopropanol (Appli. Chem., #A0900)

Kanamycin sulfate (Duchefa, #K4378)

KLORIX®, commercial sodium hypochlorite solution

Lithium Acetate (Sigma, #L-5750)

Lithium chloride (Li Cl) (Roth, #3739.1)

Magnesium chloride (Roth, #KK36.3)

Methanol (Chem Solute, #1437.2511)

Murashige and Skoog media, MS (Sigma, #M5524 and Duchefa, #M0221)

Na_2HPO_4 (Sigma, #S0876)

NaH_2PO_4 (Merck, #1.06346)

Ni-NTA Agarose (Qiagen, #139298931)

Peptone (Difco, #0122-17-4)

Phenol/Chloroform (Roth, #A156.1)

Phytoagar (Duchefa, #P0001)

Protease Inhibitors Cocktail for plant cell and tissue extracts (Sigma, #P9599)

Rifampicin (Sigma, #83907)

Sodium acetate (Merck, #1.06268)

Sodium chloride, NaCl (Merck, #1.37017)

Sodium deoxycholate (Fluka, #30970)

Sodium dodecyl sulfate, SDS (Roth, #23.26.2)

Spectinomycin (Sigma, #S-9007)

Streptomycin (Sigma, #S-9137)

Sucrose (Roth, #4621)

Tetramethylethylenediamine (TEMED) (Fluka, #87689)

Tris (hydroxymethyl) aminomethane Hydrochloride, Tris HCl (Roth, #5429.3)

Triton-X100 (Roth, #3051)

Urea (Sigma, #33247)

Yeast extract (BD, #212750)

2.1.3 Primers

Table 2.1.3-1 Primers

Primers name	Sequence
TICf	ATATGACCACATCACCTGCGGGAA
TICr	ATGGCGACAACATCGAAGGAGACT
TIC-351SAf	GTCAAATCTCGAGTCTCTGCACCGATCTCA AATCCTCAG
TIC-351SAr	GACTCCTAAACTCTAGCCACGTCTCTGAGCTCTAAACTG
TICzjf	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGGATCCGGCGAAG GAAACAGCTCCG
TICzjr	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGATTTGGGTGTACTAGT CTAAAT
TICgtf	GGTTCTGCTCTGCCACAGAGTTC
TICgtr	CTCGACTGGACAAGGTTTTGAGGA
NB4	AGCACGTGCACTCAGACACGTAC
NB2	GGTGAGTCCACAAAACATCATAG
NCED3f	CCGGTGAACTCTTCGCTTTA
NCED3r	CGACGTCCGGTGATTTAGTT
ABARf	CAAGATGGTGGCGGAGCTAG
ABARr	AATATCAATGCCAAGCGCC

2.1.4 Recipe of buffers and reagents

2.1.4.1 Seed sterilization

Bleach solution: 33% KLORIX® (v/v)

0.01% agar in sterile ddH_2O (w/v)

2.1.4.2 Growth media for plants

Table 2.1.4-1 MS media for plants

MS1	MS3
4.4 g/L MS	4.4 g/L MS
0.5 g/L MES	0.5 g/L MES
10 g/L Sucrose	30 g/L Sucrose
1.5% Phytoagar	1.5% Phytoagar
pH 5.7	pH 5.7

Table 2.1.4-2 Antibiotics for plant selection

Antibiotics	Stock	Final concentration
PPT	12 mg/mL in ddH_2O	12 μg/mL
Hygromycin	30mg/mL in ddH_2O	15 μg/mL

2.1.4.3 Bioluminescence analysis

50 mM D-luciferin stock

1 g Firefly D-Luciferin
71.3 mL 1M Triphosphate buffer (Na_2HPO_4 / NaH_2PO_4) pH 8.0

5 mM D-luciferin working solution

1.5 mL 50 mM D-luciferin stock
13.5 mL 0.01% (w/v) Triton-X100

2.1.4.4 Plant DNA extraction

TE buffer

10 mM Tris-Cl pH 8.0
1 mM EDTA

DNA Extraction Buffer (DEB)

200 mM Tris pH 8.0

240 mM NaCl
25 mM EDTA
1% (w/v) SDS

2.1.4.5 PCR

Primers (Invitrogen)

dNTP Set, 100 mM Solutions (Fermentas, # R0182)

Taq-DNA polymerase (Genaxxon Bioscience : PeqLab, #01-1000)

Pfu II Ultra® II Fusion HS DNA polymerase (Stratagene, # 600670)

QIAprep® Spin Miniprep kit (Qiagen, #27104)

10 mg/mL Ethidium bromide

6X DNA loading buffer (Fermentas, # R1151)

2X TBE Electrophoresis buffer

67.23 g/L Tris-Cl
34.31 g/L Boric acid
37.22 g/L EDTA pH 8.0

2.1.4.6 RNA extraction

RNeasy® Plant Mini Kit (Qiagen, #74904)

DNase I recombinant, RNase free (Roche, #04716728001)

Protector RNase Inhibitor (Roche, #03335402001)

2.1.4.7 Quantitative reverse transcription(qRT)-PCR

Oligo dT primer (Invitrogen)

Superscript® II reverse transcriptase (Invitrogen, #18064-014)

iQ™ SYBR® Green supermix (Bio-rad, #170-8882)

2.1.4.8 Genotyping

Taq-DNA polymerase (Genaxxon Bioscience : PeqLab, #01-1000)

SYBR green (Biotium, #31000)

2.1.4.9 Gateway cloning

GATEWAY® BP Clonase II enzyme mix (Invitrogen, 11789-020)

GATEWAY® LR Clonase II enzyme mix (Invitrogen, 11791-020)

Table 2.1.4-3 Plasmid used for molecular cloning

Plasmid	antibiotic
pDONR207	Gentamicin
pDEST22	Gentamicin
pJIC8	Carbenicillin
pGEX-6p1	Carbenicillin
pET201	Carbenicillin

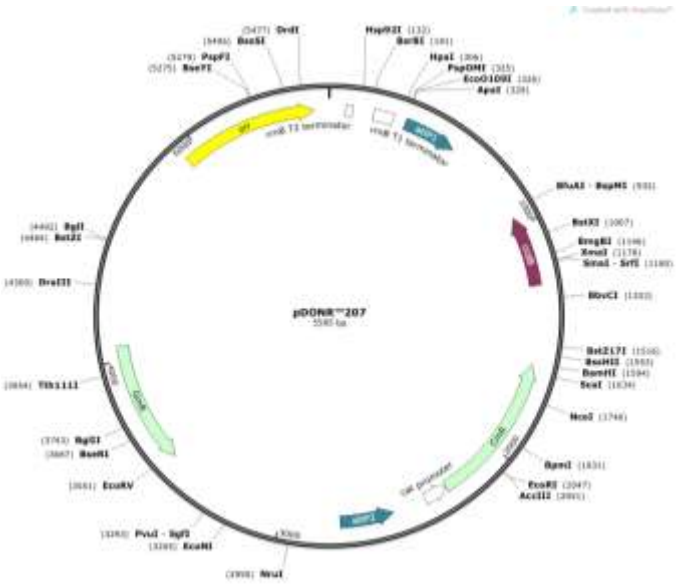


Figure 2.1.4-1 pDONR 207

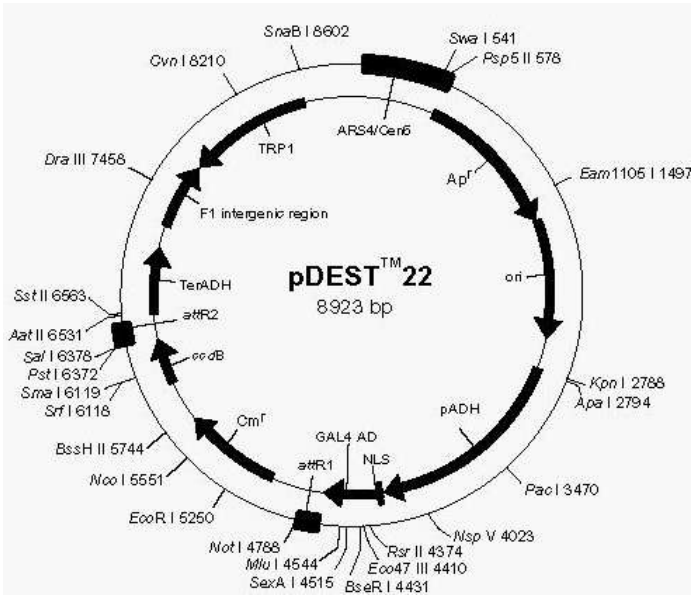


Figure 2.1.4-2 pDEST 22

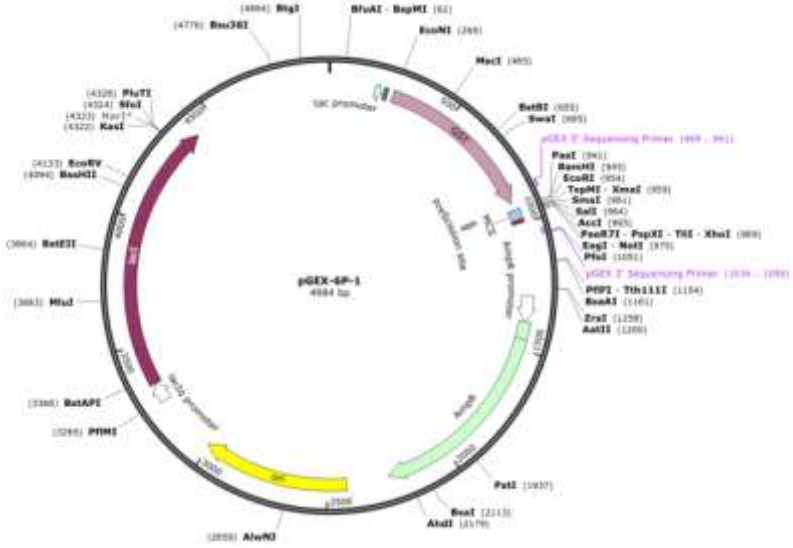


Figure 2.1.4-3 pGEX-6P-1

2.1.4.10 Growth media for bacteria

Luria Bertani (LB)

10g/L Bacto-tryptone

5g/L Yeast extract
5g/L NaCl
1% Agar
pH 7.5

YEBS

5g/L Beef extract
5g/L Peptone
5g/L Sucrose
1g/L Yeast extract
0.5g/L $MgSO_4$
1% agar
pH 7.0

Table 2.1.4-4 Antibiotics for bacteria selection

Antibiotics	Stock	Final concentration
Gentamicin	100 mg/mL in ddH_2O	10 μg /mL
Carbemicilin	100 mg/mL in ddH_2O	100 μg /mL (*E. coli*)
		50 μg/mL (*Agrobacterium*)
Kanamycin	100 mg in ddH_2O	50 μg /mL
Chloramphenicol	10 mg/mL in ethanol	30 μg /mL
Rifampicin	25 mg/mL in methanol	25 μg /mL
Spectinomycin	30 mg/mL in ddH_2O	30 μg /mL
Streptomycin	30 mg/mL in ddH_2O	30 μg /mL

2.1.4.11 Extraction and purification of *E.coli* expressed proteins

Table 2.1.4-5 Buffers for protein extraction and purification

Lysis buffer	Wash buffer	Elution buffer
50mM NaH_2PO4	50mM NaH_2PO4	50mM NaH_2PO4
300mM NaCl	300mM NaCl	300mM NaCl
10mM imidazole	20mM imidazole	250mM imidazole
pH 8.0	pH 8.0	pH 8.0

1X PBS

8g NaCl
1.54g $Na_2HPO_4 \cdot 12H_2O$
0.29g KH_2PO_4

1% Triton-X100/PBS

1% Triton-X100
1X PBS

Add ddH_2O to 1L

5mM Glutathion solution

15.4mg Glutathion powder
5ml 50mM Tris-Cl pH 8.0

Add ddH_2O to 1L

Dialysis buffer

10mM Tris-Cl pH 8.0
30% Glycerol

Add ddH_2O to 1L

2.1.4.12 *in vitro* binding buffer

Binding buffer

50mM Tris-Cl pH7.5
1mM EDTA
150mM NaCl
0.1% Triton X-100
10% Glycerol
1X BSA

Add ddH_2O to 1L

2.1.4.13 SDS-PAGE

SDS-PAGE gel

Table 2.1.4-6 Separation gel for 1.5mm case

Gel concentration	ddH2O (mL)	Seperation buffer (mL)	Acrylamide (mL)	APS (μL)	TEMED (μL)
8%	4	2	2	50	5
15%	2	2	4	50	5

Table 2.1.4-6 Stacking gel for 1.5mm case

ddH2O (ml)	Stacking buffer (ml)	Acrylamide (ml)	APS (μL)	TEMED (μL)
2.25	0.94	0.5	22.5	7.5

Separation buffer

1.5M Tris
0.4% SDS

Add ddH_2O to 1L

pH 8.8

Stacking buffer

0.5M Tris
0.4% SDS

Add ddH_2O to 1L

pH 6.8

5X SDS gel running buffer (1L)

15.17g Tris
72g glycine
5g SDS

Add ddH_2O to 1L

5X SDS sample buffer

0.225M/Tris-Cl pH 6.8
50% glycerol
5% SDS
0.25% bromophenol
0.25M DTT

Add ddH_2O to 1L

Comassie Blue staining solution

0.25g Comassie Blue

100mL of destaining solution

Destaining solution

500mL methanol
400mL ddH_2O
100mL acetic acid

Add ddH_2O to 1L

2.1.4.14 Kinase assay

5µCi γ-P^{32} ATP (Amershan-GE Healthcare Life Sciences, Germany)

Kinase buffer

20 mM HEPES
2.5 % Triton X-100
10 mM $MgCl_2$
50 mM NaF
5 mM PMSF
5X protease inhibitor cocktail

2.1.4.15 Nuclear protein extraction

Nuclei isolation buffer

50mM MES pH 8.5
25mM KCl
5mM $MgCl_2$
25g Sucrose
150ml Glycerol
10mM 2-mercaptoethanol
1mM DTT (add just before use)
1.5ml (0.3%) Triton X-100
500µl (1:1000) PIC (add just before use)
2.5 ml (1mM) PMSF (add it just before use, it is rapidly degraded)
Add Water to 500ml
pH should be around 7-8

3X washing buffer (1L)

20mM MgCl2
100mM KCl
50mM HEPES pH7.5
400g Sucrose
400 ml Glycerol
40mM 2-mercaptoethanol 2.79 ml
Add water to 1L

Before use, prepare 1X washing buffer and add 1mM DTT, 1.5mM PMSF, 0.3%

Triton X-100, 1/500 SIGMA proteinase inhibitor cocktail.

2.2 Methods

2.2.1 Seed sterilization

Seeds were placed in clean 1.5mL microcentrifuge tubes. Seeds were surface sterilized with 800µl of 100% ethanol for 2 minutes. Afterwards, ethanol was removed and seeds were rinsed with 800µl of bleach solution for 2 minutes. The bleach solution was removed and seeds were washed with 900µl of sterile water. Finally the seeds were suspended in sterile 0.01% agar water. Seeds were plated on the appropriate MS agar plate with specific antibiotics listed in Table 2.1.4-1 (if required). The plated seeds were kept at 4°C for 2-3 days in the dark, and then transferred to the growth cabinet.

2.2.2 β-estradiol treatment

In medium: Add the β-estradiol into the MS medium, made the finally concentration to 5µM, then sterilized plants on medium.

In soil: solved the β-estradiol into the water, made the finally concentration to 10µM, used the spray bottle to spray the solution on the plants.

2.2.3 Abscisic acid treatment

For analysis of Arabidopsis seedlings to ABA stress, ABA was added into the MS medium, made the finally concentration to 5µM and made them to ABA medium plate. The Arabidopsis seedlings were germinated and grew on the normal MS plate for 1 week, thus, transferred to the ABA medium plate, continued to grow for 1 week.

2.2.4 Drought treatment assay

For measure drought tolerance in different plant lines, I defined the survival rate (Survival rate = (Number of surviving plants / Number of all plants)%) after drought

treatment to be a standard of drought resistance. The detail steps are as follow:

1. The seeds were stratified at 4°C for 2-3 days after sterilization, then transferred to soil pots for about two weeks.
2. Water treatment was stopped the plants until wild type plants were dead. Watering pots was resumed for a further week. Plants that survived were then calculated with the total sown for calculations.

2.2.5 Anthocyanin measurement

Anthocyanin was extracted by Arabidopsis grown on MS1 medium for 2 weeks. The extraction and measurement method was as described by Teramura et al. (1984) [109]. The detail operating steps are as follow:

1. Transferred plants from growth medium. Measure the weight of the plants (basically pick up 500mg). Plant was speed frozen by the liquid N2 and pulverized with a mortar and pestle.
2. Transferred the powder into 15ml tube. Add as equal volume of extraction buffer (Volume fraction of 19% propanol, 1% HCl in ddH_2O). Allow extraction to occur overnight in the dark at 4°C.
3. Centrifuged the tube and transfer the supernatant to a fresh tube.
4. Put the supernatant into cuvette. Measurement the anthocyanin concentration with the UV spectrophotometer in OD530. Ten times the result of OD530, the finally result is the number of “anthocyanin unite”. (0.1 of OD530 is one Anthocyanin unite)

The formula of the finally anthocyanin concentration calculation:

Anthocyanin concentration (anthocyanin unite) = 10×OD530

2.2.6 Chlorophyll and measurement

Chlorophyll measurement was performed according to Contillo et al. (1996). Plants were grown for 3 weeks under long-day (16 h light/8 h dark) conditions [110], and the third leaf was subjected to determine the chlorophyll level using the Konica

Minolta SPAD-502.

2.2.7 Leaf length-to-width measurement

For determining leaf length-to-width ratio, the third true leaf was picked and used the ruler measured the length and width three times, thus, the leaf length-to-width ratio was calculated by the calculation:

length-to-width ratio = length/width

2.2.8 Bioluminescence

Plant lines containing a construct of circadian promoter fused to luciferase gene were used to study clock periodicity by measurement of bioluminescence with a TOPCOUNT scintillation counter from Perkin Elmer. The promoter linking with luciferase constructs used are listed in table 2 in this section. This protocol is according to Gould et al. (2006) [111].

1. The experiments used 7days-old Arabidopsis seedlings growing under 12:12 LD light condition, and transferring to previously ethanol sterilized and dried 96well black microtiter plates (Perkin-Elmer), containing MS3 media with or without necessary chemical or hormone, depended by experiment.
2. Set up between 24 and 48 seedlings per genotype, and transfer only one seedling to a single well. Add 15 µl of 5 mM luciferin which was dissolved in 0.1% Triton X-100 ddH_2O, to each well. The plate was sealed with transparent film.
3. Used the top of tweezers to pierce a hole for each well which was perforated for allowing gas exchange. Placed all the plates in the growth chamber for adapt to the environment over night.
4. Placed the plates in a TOPCOUNT with trichromatic LED panels having red and blue light at a fluence of 1-1.5 µE each as a light source. For this, the plates were stacked by alternating the experimental set up with reflector plates consisting of a mirror that reflects the light source in an equal distribution.
5. The TOPCOUNT machine measured each plate after 1 minute of delay for avoiding chlorophyll autofluorescence and by reading each well for 5 seconds.

The plates were entrained for 1 or 2 days in the topcount under red and blue light for 12 hours and 12 hours of darkness before setting the experiment to constant light (LL).

2.2.9 Analysis of clock rhythms

For visualization of the luminescence, I used the software TOPTEMP II macro in EXCEL (available under the website http://millar.bio.ed.ac.uk/Downloads.html) to analysis the data from the scintillation counter. The final figures showed the period condition was performed by the software named Biological Analysis Software System (BRASS) macro in Excel. This software includes the Fast Fourier Transformation Non Linear Least Square (FFT-NLLS) analysis tool [112]. Period analysis was performed for 72 hours after the start of free run. The standard analysis parameters counted the period, which was between 15-35 hours with a confidence probability of 95%. Rhythms were assessed by comparison of Relative Amplitude of Error (RAE) weighted means of the period lengths (data from BRASS) in addition to comparisons of individual period and of RAE values. The RAE is a ratio of the amplitude's error in relation to an estimate of the most probable amplitude that describes the fit of the actual data to a theoretical cosine curve. Therefore, the RAE is a measure to evaluate rhythmicity, where a RAE equal to 0 is a perfect cosine curve and RAE equal to 1 is arrhythmic.

2.2.10 Plant DNA extraction

In order to extract DNA from Arabidopsis, the procedure was as follow:

1. The plant tissue was harvested in 1.5mL microcentrifuge tube with 2 steelballs, frozen in liquid nitrogen and vortexed to powder.
2. Add 100µl DNA extraction buffer (DEB) and ground at room temperature (RT). Then, an additional 400µl of DEB and 100µl chloroform was added, followed by a 5-minute vortex.

3. The tubes were centrifuged in 14000 rpm for 10 minutes. A total of 350µl of supernatant was transferred to a new tube and mixed with an equal volume of isopropanol.
4. Another 10-minute centrifuge and the supernatant were discarded. The pellet was rinsed by 500µl 70% (v/v) ethanol. Ethanol was then removed after another 5-minute centrifuge (14,000rpm).
5. The pellet was air-dried and resuspended in 100µl 1X TE buffer. Additionally, the concentration of DNA was measured by NanoDrop 1000 spectrophotometer (Peqlab).

2.2.11 Genotyping

To genotype Arabidopsis, genomic DNA was extracted from individual seedlings. The following mixtures were prepared before for melt curve genotyping in the LightCycler 480 II (Roche):

PCR reaction mixture
(for 1 reaction)

ddH_2O	3.6µl
SYBR Green supermix	5µl
Primer (f)	0.2µl
Primer (r)	0.2µl
DNA	1µl
Total	10µl

Program setting and data analysis followed the Manual of LightCycler 480 II (Roche).

To genotype *tic*-1 Arabidopsis mutants, genomic DNA was extracted from individual *tic*-1 seedlings. The following mixtures were prepared before running PCR.

PCR reaction mixture

(for 1 reaction)

10x Taq DNA polymerase buffer	2.0µl
dNTP	2.0µl
DNA	0.5µl
Primer mix	2.0µl
Taq DNA polymerase	0.2µl
ddH_2O	13.3µl
Total	20.0µl

Primers used for PCR are TICgtf/r in Table 2.9.

After PCR, the PCR production added the restriction enzymes E.Col I (HF) and incubated 2 hours in 37 °C, and run the gel, the wild type lines had a bigger size band and *tic*-1 homozygous lines had a smaller size band, the heterozygosis *tic*-1 lines had two different size bands.

2.2.12 RNA extraction

For extract Arabidopsis RNA, the seedlings were grown for 7 days. Around 100 mg seedlings were harvested and the extraction steps are as follow:

1. Transferred the seedlings to a 1.5mL microcentrifuge tube with two steel balls. The tube was frozen in liquid nitrogen immediately and uses the vortex to break them to powder.
2. Use the RNeasy® Plant Mini Kit was used to extract RNA. In the end, the RNA was resuspended in 80µl RNase-free water.
3. Before performing DNA digestion, resuspended RNA was centrifuged at 14,000rpm at 4°C for 30 minutes. Then, 70µl of the supernatant was transferred to a fresh tube. To do this, 2µl DNase, 1µl RNase inhibitor and 8µl DNase recombinant buffer were added. This was incubated at 37°C for 2 hours.
4. Subsequently, RNA was precipitated by adding 8µl 3M NaAc (pH 5.2) and 160µl 100% ethanol. The precipitation lasted overnight at -20°C. RNA was collected by centrifuging at 1400rpm at 4°C for 1 hour. The supernatant was removed and the pellet was washed three times with 100% ethanol. In the end, the pellet was

resuspended in 32µl RNase-free water. From this 32µl RNA, 1µl was used to measure the RNA concentration by using NanoDrop 1000 and 1µl was used as template to check for genomic DNA contamination.

5. Used 1µl RNA with primer NB4/2 used the PCR method to detect the DNA contamination, the PCR program is 95°C 5 minutes initial denaturation, 30 times of 95°C 30 seconds denaturation 55°C 30 seconds annealing and 72°C 30 seconds extending, 72°C 7 minutes total extending, 4°C stocking. Following mixtures were prepared before running PCR. If there was no DNA contamination, the RNA sample could not have production in this reaction and could not show any bands on the electrophoresis.

PCR reaction mixture

(for 1 reaction)

10x Taq DNA polymerase buffer	2.0µl
dNTP	2.0µl
RNA	1.0µl
Primer mix	2.0µl
Taq DNA polymerase	0.2µl
ddH_2O	12.8µl
Total	20.0µl

Primers used for PCR are NB4/2 in Table 2.9.

2.2.13 Reverse transcription

cDNA was generated by RNA reverse transcription. Before reverse transcription, RNA concentration was measured and 4µg RNA was taken for reverse transcription. Reverse transcription was performed, according to cDNA synthesis protocol for Superscript® II RT (Invitrogen).

2.2.14 qRT-PCR

Enrichment of DNA sequences was measured by qRT-PCR. Primers were designed using to the net web service http://sg.idtdna.com/Primerquest/Home/Index, obtain amplicon sizes that ranged from 150-200bp. qRT-PCR was performed with iQ™ SYBR® Green supermix (Bio-rad) on the LightCycler 480 II (Roche).

2.2.15 Nuclear protein extraction

For extraction of nuclear protein, the plant seedlings were harvested and dried with paper and frozen in liquid nitrogen. (The seedlings should be 7-15 days old). Grind the tissue in a big mortar and a pestle with liquid nitrogen until obtaining a fine powder (about 10ml powder is obtained). The powder can be kept in -80°C (until protein extraction is carried out). The extraction steps are as follow:

1. Adjust the centrifuge to 4°C, brought some liquid nitrogen and prepare breaking, washing and TALON buffers. Took a big mortar and a pestle and fill it in with liquid nitrogen (to the top). Added the frozen seedlings powder and slowly 20ml (or more, depends on the quantity of powder) of nuclei breaking buffer (break the bubbles as they were forming with the pestle, until getting a fine powder).
2. Let the extract thaw in the mortar until it becomes liquid again, then, passed the extract through miracloth on the top of a funnel into 15ml falcon tube (or 50ml, depends on the quantity of buffer I used before). Kept on ice. Centrifuged at 4000 rpm for 15 min at 40°C. Discarded quickly the supernatant (can be kept for the cytosolic fraction). Resuspend the pellet in 10ml 1X washing buffer. Centrifuge at 3000 rpm for 10 min at 4°C. Quickly discarded the supernatant.
3. Resuspended the pellet in 10ml 1X washing buffer. Centrifuged at 2500 rpm for 10 min at 4°C. Discarded quickly the supernatant. Resuspended in 3ml 1X washing buffer and then transferred into two 2ml microcentrifuge tubes (1 can be kept in -20°C). Centrifuged at 4000 rpm for 10 min at 4°C. (In the small centrifuge). Discard quickly the supernatant.
4. Added between 300-500µl of TALON denature buffer (depending on the size of the pellet). Incubated over-night on a rolling platform. Place at room temperature for about 15 min. Centrifuged at 4000 rpm for 15 min (in RT). Collect the supernatant (this is the nuclear extract). This can be kept several weeks at -20°C and several months at -80°C. (The pellet at this stage shouldn`t be green, the supernatant should be a bit brownish).

2.2.16 Gene cloning

For the PCR amplification of the *TIC* and the genes, the PCR mixture was prepared as follow:

10x Pfu Ultra II buffer	5.0µl
dNTP	5.0µl
DNA	1.0µl
Primer mix	2.0µl
Pfu Ultral II DNA polymerase	1.0µl
H_2O	36.0µl
Total	50.0µl

Primers used for PCR are listed in Table 2.9.

2.2.17 Cloning with Gateway

All Gateway® empty vectors were propagated in *Escherichia coli (E. coli) DB3.1* cells. *E. coli DH5α* cells were used to propagate transformed vectors. BP reaction was performed to recombine PCR products into pDONR201. The BP reaction was set up as follow:

Target DNA	0.5µl (≈100fmol)
pDONR201	0.5µl (≈100fmol)
TE buffer	3µl
BP Clonase Enzyme mix	1µl
Total	5µl

The reaction was left at 25°C for at least 6 hours. After that, 1µl of this reaction mix was used to transform *E. coli DH5α* cells.

LR reaction was performed to transfer target genes from entry vectors to destination vectors. LR reaction was set up as follow:

pDONR201	0.5µl
Destination vector	0.5µl
TE buffer	3µl
LR Clonase Enzyme mix	1µl
Total	5µl

The reaction was left at 25°C for at least 6 hours. Then, 1µl of LR reaction was used to transform *E. coli DH5α* cells.

2.2.18 *E.coli* transformation

For *E. coli* transformation, an aliquot (50µl) of chemical-competent *E. coli* cells was thawed on ice and 1µl of plasmid was added to the cells. After being left on ice for 30 minutes, the cells were heated at 42°C for 1-2 minutes. Then the cells were immediately moved onto ice and cooled for 2 minutes. After that, 500µl of LB media was added to the cells, and they were incubated at 37°C for 1 hour, with gentle shaking. After incubation, 100µl of the cell suspension was plated on an appropriate selective LB agar plate. Plates were sealed with parafilm and incubated overnight at a 37°C.

2.2.19 Isolation of Plasmid DNA

A single colony growing on the selective plate was picked and inoculated in 10mL of selective LB media. The cells were cultured at 37°C for approximately 16 hours. Then the cells were collected by centrifuging at 4000rpm for 10 minutes. Plasmid was extracted by using the Qiaprep® Spin Miniprep Kit (Qiagen). Finally, DNA concentration was measured by NanoDrop 1000 spectrophotometer (Peqlab).

2.2.20 Mutagenesis

The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers:

1. Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
2. Primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78°C. Primers longer than 45 bases may be used, but using longer

primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. (Fig 2.2.20).

3. The desired mutation (deletion or insertion) should be in the middle of the primer with 10–15 bases of correct sequence on both sides. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

The mutant strand synthesis reaction (Thermal Cycling) was used with normal PCR machine. The reaction system is as follow:

2 μl of 10× Pfu Ultral II DNA polymerase buffer (see Preparation of Media and Reagents)

0.4 μl of pDEST32 plasmid (5 ng/μl)

1 μl (100 ng) of primer1

1 μl (100 ng) of primer2

1 μl of dNTP

0.2μl of Pfu Ultral II DNA polymerase

Added double-distilled water (ddH2O) to a final volume of 20 μl

The transferred sample on PCR machine, the PCR program is as follow:

1. 95°C 30 seconds

2. 95°C 30 seconds

3. 55°C 1 minute

4. 68°C 15 minute

Step 2 to 4 repeat 18 times

5. 10°C storage

Added 1μl DpnI restriction enzyme and 37°C digest 2 hours.

DNA after digestion was transformed into E. coli competent cells. Then the correct site-directed mutagenesis of the plasmid can be got by antibiotic selection.

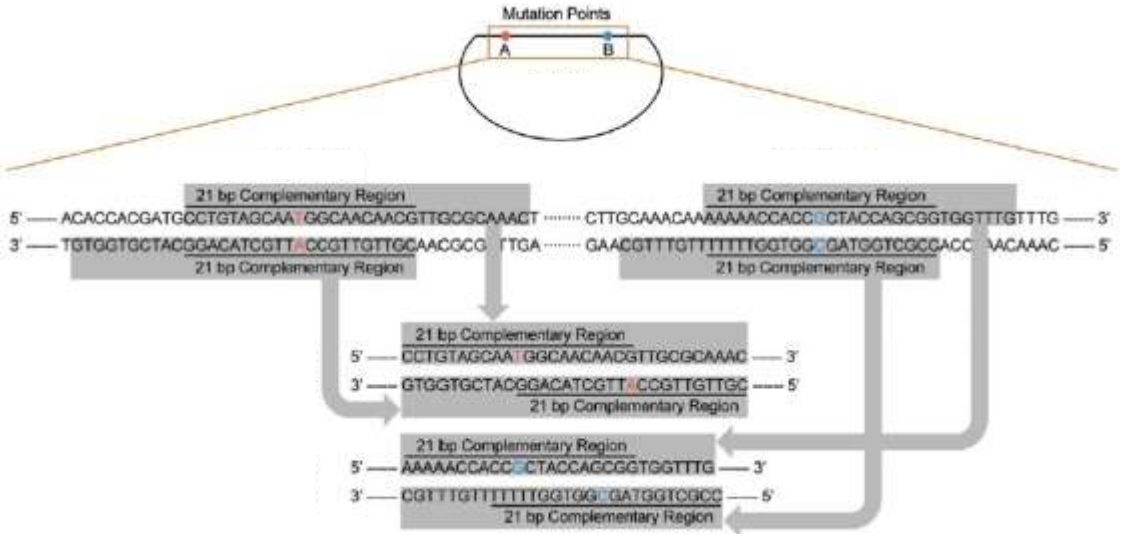


Figure 2.2.20 Oligonucleotide design for the PCR amplifications used in the mutagenesis method, pick the mutant base as center and extend 12-22 bases for each side. The primers' total length is about 25-45 bases. In each mutagenesis site, there are two primers which have same sequence and 3', 5' opposite direction.

2.2.21 PIPE

The PIPE method is based on the observation that, contrary to popular assumption, normal PCR amplifications result in mixtures of products, which are not fully double stranded. The 5′ends of such products are left variably unpaired by incomplete 5′→ 3′primer extension caused by sequence-specific stalling and changes in the reaction equilibrium (less dNTPs available, more template copies to synthesize) in the final cycles of PCR. These unpaired 5′ ends on the PCR products are the same 5′ ends on the synthetic amplification primers. Therefore, a simple oligonucleotide design rule can control the sequences of these ends in a way that promotes easy cloning and mutagenesis.

Because the V-PIPE (Vector PIPE) of fragment deletion is only used in this thesis. Therefore, only introduce the V-PIPE method, the steps are as follow:

1. Primer design: The first 15 bases on the 5′ ends of the primers were designed to be directionally complementary such that the resultant PCR fragment(s) can anneal as desired and become viable plasmids upon transformation. In basic PIPE cloning,

the vector was linearized by V-PIPE PCR amplification and contains two distinct 5′ ends. The detail is following the figure (Fig 2.2.21).

2. PCR: The reaction system is as follow:

2 μl of 10×KOD-DNA polymerase buffer

0.4 μl of plasmid (5 ng/μl)

1 μl (100 ng) of primer1

1 μl (100 ng) of primer2

1 μl of dNTP

0.2μl of KOD-DNA polymerase

Add double-distilled water (ddH2O) to a final volume of 20 μl

Then transfer sample on PCR machine, the PCR program is as follow:

1. 95°C 2 min

2. 95°C 30 s

3. 55°C 45 s

4. 68°C 14 min,

Step 2 to 4 repeat 25 times

5. 10°C storage

Add 1μl DpnI restriction enzyme and 37°C digest 2 hours.

Therefor, the DNA after digestion was transformed into E. coli competent cells. Then the correct site-directed mutagenesis of the plasmid could be got by antibiotic selection.

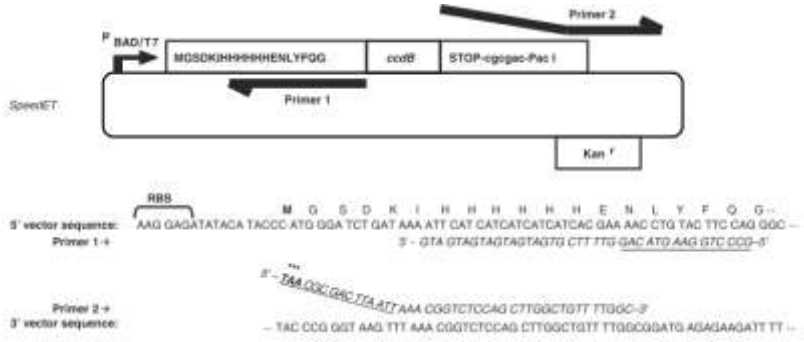


Figure 2.2.21 Oligonucleotide design for the PCR amplifications used in the PIPE method, the 15 base complementary overlaps are shown as the underlined portion of each primer sequence. Primers 1 and 2 are examples of primers that could be used to PCR amplify a vector.

2.2.22 Isolation and purification of proteins

The protein expression was in *E. Coli* BL21 (DE3), and the method is as follow:

1. Growing 10mL E. Coli cells were pre-cultured in selective LB media overnight. From 5mL culture, 1mL of cell culture was added to 300mL fresh selective LB medium.
2. The new culture was subsequently incubated at 37°C until its OD600 reached 0.6-0.8, added IPTG to induce protein expression (1mM as final concentration). The cell culture was incubated at 16°C overnight.
3. On the next day, cells were collected by centrifuging at 4000rpm for 10 minutes. Use the ddH_2O to wash the cells once.

To isolate the protein, I used two different tags, firstly introduce the His tag protein purification, the steps are as follow:

1. Used 10mL lysis buffer together with 1 piece of proteinase inhibitor to add into the cells and these cells were broken by sonification. After that, particulate material was pelleted by centrifugation at 13,000rpm for 30 minutes.
2. Picked up 1mL Ni-NTA Agarose which was rinsed by 10mL lysis buffer for 3 times in 15mL falcon tube. After centrifuging, the clarified supernatant was loaded onto the Ni-NTA Agarose beads.
3. For maximal binding of the His- tagged protein, the tube was rotated for 2 hours at 4°C. The fully bound Ni-NTA Agarose beads were spun down at 500rpm for 2 minutes and the supernatant was removed.
4. The Ni-NTA Agarose beads were twice rinsed by 10mL wash buffer and resuspended in 10mL wash buffer before loaded onto an empty column. The flow through wash buffer was discarded. In the end, the His-tagged protein was eluted by 10mL elution buffer.
5. Run the solution of elution buffer into the PAGE gel and Coomassie blue staining; pick the sample which has the correct band, frozen in -20°C.

The steps of GST tag protein purification are as follow:

1. Use the 1X PBS wash the cells and centrifuged. The pellet was resuspended in 10mL of 1X PBS. Afterwards, 100μl of lysozyme (100 mg/mL) was added, followed by a 15-minute incubation on ice.
2. Added 1% of Triton X-100/PBS and centrifuged at 10000rpm at 4°C for 20 minutes after rocked at 4°C for 30 minutes. During the centrifuge, 100μl of glutathione sepharose 4B resin was prepared and equilibrated with 1X PBS three times.
3. After centrifuge, the clarified supernatant was rocked with pre-equilibrated resin at 4°C for 2 hours. The resin was collected by centrifuging at 1500rpm for 5 minutes and washed by 1X PBS. In the end, the GST-tagged protein was eluted with 10mM glutathione solutions.
4. Run the solution of elution buffer into the PAGE gel and Coomassie blue staining; pick the sample which has the correct band, frozen in -20°C.

2.2.23 *in vitro* protein binding assay

To identify if proteins interacted with each other *in vitro*, 2μg His-tagged protein were mixed with 2μg GST-tagged protein, binding buffer and Ni-NTA agarose, and then was incubated at 4°C for 2 hours. The Ni-NTA agarose was spun down at 8000rpm at 4°C and washed with 400μl of binding buffer. This was repeated 4 times. In the end, 50μl 1X SDS sample buffer was added and boiled at 95°C for 5 minutes. This sample was ready for SDS-PAGE.

2.2.24 Kinase assay

For detect the phosphorylation between AKIN10 and TIC I used the protein which is purified from *E. Coli*, the kinase phosphorylation reaction experiment steps are as follow:

1. Equal amounts of His-AKIN10 and GST-TIC fragment described above were mixed and labeled with 5μCi γ-32ATP in 1X kinase buffer using as a control a

separate sample of His-AKIN10 without substrate and TIC fragment without kinase.

2. The kinase reaction was performed at room temperature and stopped by addition of 4X SDS loading buffer added with 100 mM EDTA. The samples were resolved by SDS-PAGE electrophoresis.
3. The gel was dried between sheets of Whatman paper under vacuum and finally exposed to a film for autoradiography [113].

2.2.25 Agrobacterium transformation

Agrobacterium tumefaciens (*Agrobacterium*) strain ABI [114] was used. An aliquot (50µl) of electrocompetent cells was thawed on ice. Then, 1µl of plasmid and 80µl of sterile ddH_2O were added to the cells. The diluted cells were transferred to an electroporation cuvette for electroporation. After electroporation, cells were immediately mixed with 900µl of LB media and transferred to 1.5mL microcentrifuge tube. After incubation for 2 hours at 28°C, 100µl of the cell culture was plated on selective YEBS agar plate. Plates were sealed with parafilm and incubated at 28°C for 2 days to allow for colony growth [115].

3 Results and discussion

3.1 Overexpression of TIME FOR COFFEE results in Similar Phenotypes in Diverse Growth and Physiological Responses

3.1.1 TIC overexpression line plants may also have circadian rhythms and other phenotypes

Due to the importance of TIC function in the altering of the circadian clock and other physiological responses, I investigated the functional changes of different TIC expression in vivo. Currently studies only show that TIC is composed of 1555 amino acids. No known functional motif can be found in its primary structure. In a previous study, the TIC loss-of-function mutant *tic*-1 was showed to have a short circadian period phenotype in Ws background and no other result of different condition for any TIC expression model.

Integration of external signals by this clock ensures metabolic homeostasis and ultimately enhances fitness. TIME FOR COFFEE (TIC) was known to be associated to the circadian clock, being required to maintain rhythmic period and amplitude, and to regulate clock-driven physiological responses. The molecular function of TIC has so far only been studied with loss-of-function mutants. The biochemical activity of TIC remains elusive, but the condition of rhythmic period in TIC overexpression lines is still unknown. Previous research of rhythmic period and amplitude of the tic mutant is only in Ws background, and the condition in Colombia background is still unknown.

To learn more about TIC in diverse physiological processes, I made TIC overexpressing plants (TICox) lines and characterized their impact on plant growth, development, and circadian clock activity. TICox plants displayed phenotypic similarity with tic mutants. This included defects in leaf morphology, the developmental transition from the vegetative to reproductive phase, and circadian clock function. These observations allowed me to hypothesize that TIC is an element

of protein complexes that are involved in global biological processes.

3.1.2 The lower leaf length-to-width ratio phenotype in TIC Overexpression Plants

Towards investigating the molecular and biochemical function of TIC, I first generated TIC overexpression (TICox) plants in wild-type Columbia (Col) background. TAP-tag was fused to the 5' end of TIC full-length cDNA, and the construct was expressed under control of the CaMV 35S promoter (Figure 3.1.2-1 A). We determined TIC transcript accumulation in homozygous transgenic plants, and selected two independent lines with the highest expression, namely TICox-8 and TICox-11. In qRT-PCR analysis, TIC transcript level was shown to be elevated about 15 fold and 21 fold in TICox-8 and TICox-11, respectively, compared to the wild type (Figure 3.1.2-1 B).

The generated transgenic plants are TIC overexpressing lines. In previous studies, *tic*2 mutants displayed slower development compared to wild type (leaves numbers less than wildtype). This result indicated TIC could be involved in altering leaf morphology and pigment biosynthesis. Thus, I measured leaf morphology of TICox plants, and compared them to *tic*2. The third leaves of three week old wild-type plants displayed 1.57±0.12 (±SD) length-to-width (L/W) ratio and the *tic*-2 mutant displayed 1.10±0.10 L/W ratio (P-value: 1.42E-10). *tic* generates round shaped leaves and this can be observed consistently, when considering leaf length compared to width. Surprisingly, TICox plants showed a similar shape as *tic*-2 (Figure 3.1.2-1 C), with the ratio measurement of TICox-8 and TICox-11, these two lines displayed 1.26±0.11 and 1.33±0.19 L/W ratio, respectively (P-value: 6.72E-7 (TICox-8), 0.001 (TICox-11)) (Figure 3.1.2-1 C). *tic*-2 is known to have serrated leaves, and this could be also seen in both TICox-8 and TICox-11 plants (Figure 3.1.2-1 D).

Next, I investigated developmental traits of TICox and *tic*-2. TICox plants displayed developmental defects similar to *tic*-2, such as delayed transition from vegetative to reproductive phase under inductive long-day conditions (Figure 3.1.2-1

E). Wild type plants produced 16.6±1.45 (±SD) total leaves until the first flower opened, but *tic*-2 plants flowered after producing 41.5±2.02 leaves (P-value: 2.04E-23). TICox-8 and TICox-11 flowered when plants generated 30.6±1.91 and 27.1±2.31 leaves, respectively (P-value: 4.27E-17 (TICox-8), 2.86E-12 (TICox-11)) (Figure 3.1.2-1 E), consistently supporting above observations that TICox plants similarly behave to tic mutants albeit the response is marginally attenuated.

Unlike with other observed phenotypes, chlorophyll a+b accumulation in TICox plants was comparable with the wild type (Figure 3.1.2-1 F), whereas it was significantly elevated in *tic*-2. The observed TICox phenotypes, such as round leaf shape and delayed flowering, already appeared in the T1 transgenic plant stage, indicating dominant effects of the construct. In contrast, similar phenotypes of *tic* mutants can only be seen in homozygous mutant plants, reflecting the recessive mutant allele. These results collectively indicate that TICox plants are developmentally and morphologically comparable with *tic*-2, but their phenotypic strength was slightly reduced compared to *tic*-2.

3.1.3 TICox Plants Display a Short Circadian Period

I next tested if overexpression of TIC caused altered circadian clock activity. Since both TICox-8 and TICox-11 transgenic lines expressed similarly high levels of TIC mRNA, and displayed largely identical phenotypes, we performed this analysis only with TICox-11 line. To monitor promoter activity of the circadian clock morning-expressed gene LHY, we introduced a construct with the LHY promoter fused to the luciferase (LHY::LUC) into TICox-11, and performed a luciferase reporter assay. Plants were entrained under 12 h light / 12 h dark (12L/12D) conditions for 8 days, then transferred into constant red and blue light (R+B) conditions. The rhythmic luciferase activity was monitored over 4 days under constant light conditions, and then, circadian period was analyzed. TICox-11 plants displayed a significantly shortened period under constant R+B conditions; the TICox-11 plants displayed a 22.0 h±0.14 h (±SEM) period, whereas it was 24.7 h±0.11 h (±SEM) in

the wild type (P-value: 3.24E-22) (Figure 2.1.3-1 A, Figure 2.1.3-2 B, Figure 2.1.3-2 E).

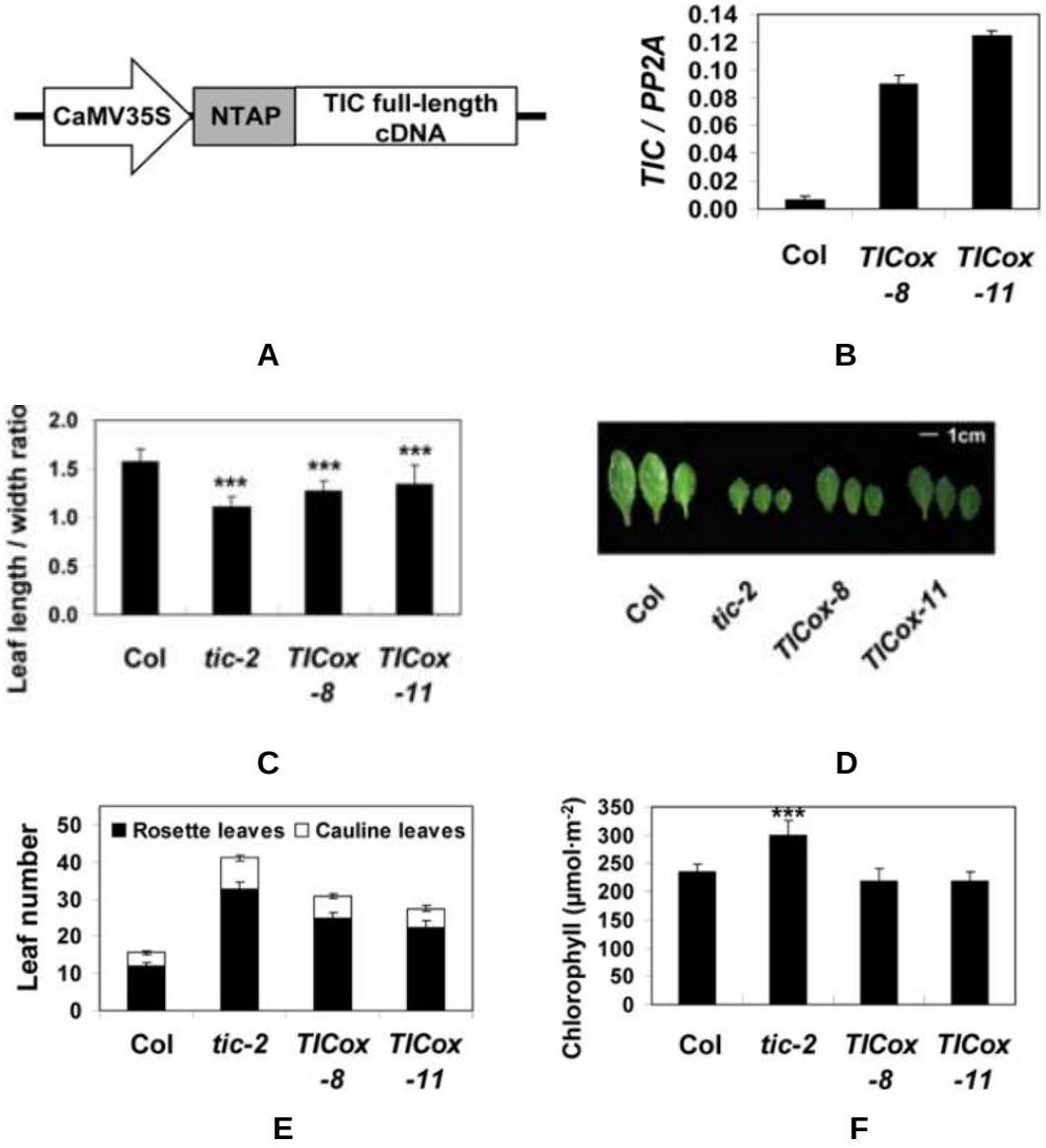


Figure 3.1.2-1. TICox plants display similar phenotype with tic-2 mutants.

A. DNA-construct model for generating TICox transgenic plants that express TAP-TIC under the control of CaMV 35S promoter.

B. Quantitative RT-PCR of TIC relative to PP2A. Col, TICox-8, and TICox-11 plants were grown for 7 days and total RNA was extracted. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate standard deviation.

C. Leaf morphology levels. and chlorophyll a+b levels. Col, tic-2, TICox-8, and TICox-11 plants were grown under long days (16 h light / 8 h dark) for 3 weeks. For leaf length-to-width ratio

D. Leaf morphology of Col, tic-2, TICox-8, and TICox-11 plants grown under long-day conditions for 3 weeks.

E. Flowering time determination by counting rosette and cauline leaves when the first flower opened. Plants were grown under long-day conditions, and leaf number was counted from more

than 9 independent plants. Error bars indicate standard deviation. Asterisks indicate statistically significant differences compared with the wild type (*** P-value < 0.001).
F. the third true leaf was measured from 13 independent plants, and error bars indicate standard deviation.

The biological rhythm of TICox plants was well maintained without causing premature dampening of the oscillation (Figure 2.1.3-1 A). Furthermore, the rhythm of each individual plant was robust, as all plants displayed a relative amplitude error (RAE) below 0.6 (Figure 2.1.3-1 B). This short period phenotype of TICox is comparable with that of tic mutants, whose rhythmic period was shortened (Hall, Bastow et al. 2003; Ding, Millar et al. 2007). A similar period shortening effect of TIC overexpression was further observed in constant darkness. Col plants showed a 26.7 h±0.28 h (±SEM) period, whereas TICox-11 showed a 24.2 h±0.39 h (±SEM) period (P-value: 5.6E-4) (Figure 2.1.3-1 C-E). This period shortening effect of overexpressed TIC was further observed under constant monochromatic red (Rc) or blue (Bc) light conditions, and the period between the wild type and TICox were statistically significantly different (Figure 2.1.3-1 E). Collectively, elevation of TIC expression shortened the circadian period regardless of the ambient light conditions. Therefore, TICox plants have similar defects in their circadian clock as *tic*-2.

3.1.4 Discussion

TIC is known to be required for proper clock activity, such as maintaining circadian period and amplitude, and regulating clock-driven output responses [12,116]. However, the exact molecular and biochemical function of TIC has not been clearly defined. In this study, we generated TICox plants and characterized their growth and developmental phenotypes, as well as circadian-clock traits to start to create a resource to understand the molecular nature of TIC.

I found that TICox plants displayed comparable growth, developmental, and physiological phenotypes as tic mutants. TICox produced round shaped serrated leaves, and flowered significantly later than the wild type, which is similar to *tic*-2 (Figure 2.1.2-1). In that TIC contributes to hormone responses, I note that disparate

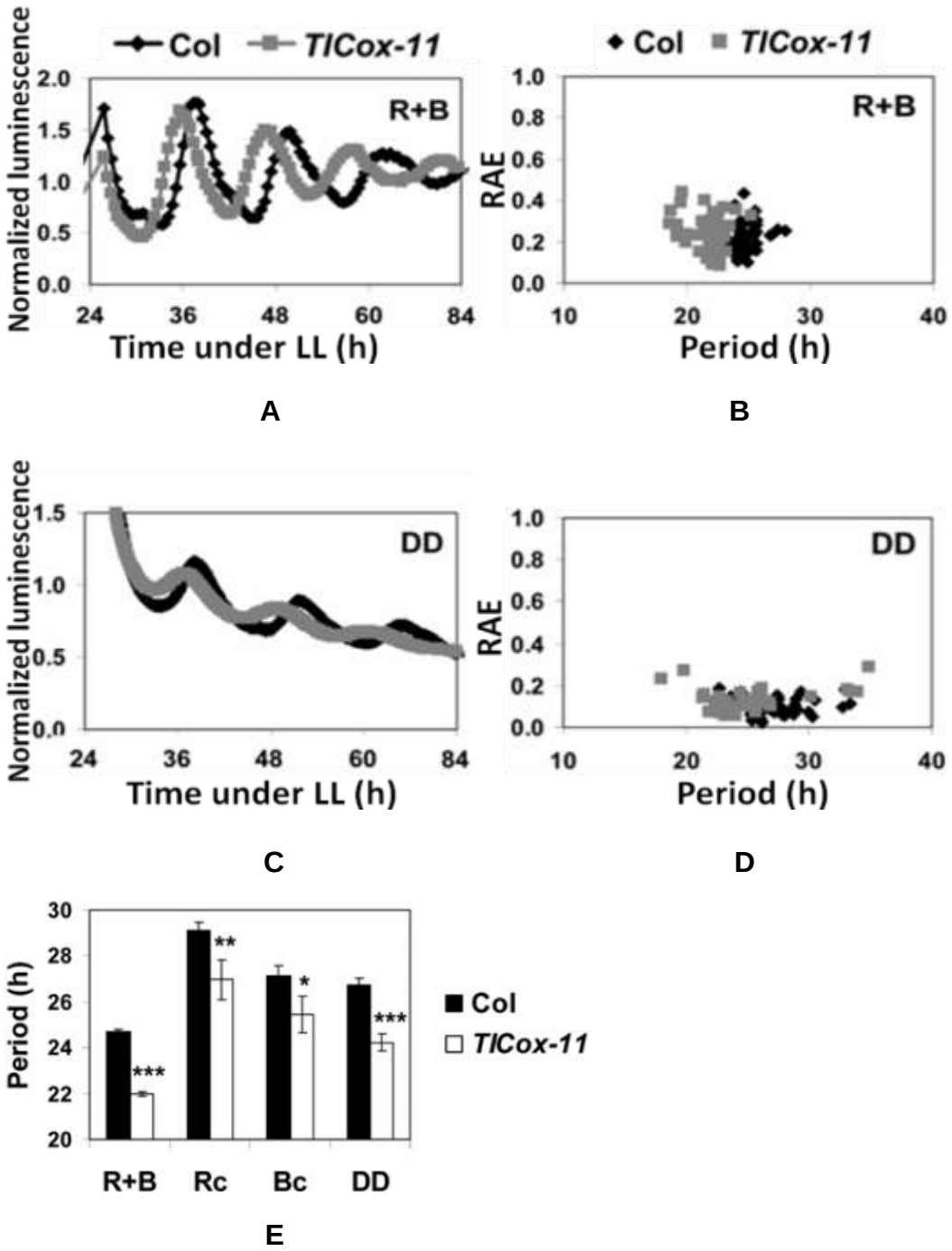


Figure 3.1.3-1 TICox displays significantly shorter circadian period, compared to Col. Wild type and TICox-11 plants harboring LHY::LUC construct were entrained under 12h light/12h dark conditions for 8 days, and transferred into constant light or dark

A. B. Normalized bioluminescence of LHY::LUC and Period versus relative amplitude error (RAE) of individual plants in light. (LL)

C. D. Normalized bioluminescence of LHY::LUC and Period versus relative amplitude error (RAE) of individual plants in dark. (DD)

E. Period versus light conditions and ecotypes. Error bars indicate standard error. Asterisks indicate statistically significant differences compared with Col (* P-value < 0.05, ** P-value < 0.01, and *** P-value < 0.001).

hormone pathways converge in flowering-time regulation [117]. In addition, TICox plants displayed a short circadian period, which was also comparable with that seen in *tic*-2 (Figure 2.1.3-1). Therefore, TICox and *tic*-2 consistently resembled each other in various phenotypic aspects.

In previous studies, 35S::GFP-TIC construct was shown to complement *tic*-1 mutant phenotype [11]. Considering the phenotypic resemblance of TICox and *tic*-2 in this study, and phenotypic complementation of *tic*-1 by 35S::GFP-TIC ina previously published result, it seems that TIC is functional only when it is expressed within a biologically relevant range. We hypothesize that TIC might form an important heteromeric protein complex and titration of TIC is critical for the biochemical action of this complex (Figure 2.1.4-1). Both lack and over-accumulation of TIC might prevent formation of physiologically functional complexes, either by the lack of TIC protein, or by titrating out relevant inter-acting proteins.

In a previous study, it was shown that LHY expression is repressed in *tic*-1, a mutant allele of TIC in the Wassilewskija (WS) ecotype [12]. We could show here that LHY is rhythmically expressed in *tic*-2, another allele in Col ecotype (Figure 2.1.3-1). The discrepancy between *tic*-1 and *tic*2 in expressing LHY might be caused by the difference of ecotypes between two studies. The protein instability of the overexpressed TIC further suggests that TIC is possibly involved in the protein degradation machinery that recruits target proteins to be degraded together with TIC. Indeed, TIC was shown to be required to degrade its direct protein-protein interaction partner MYC2 [116].

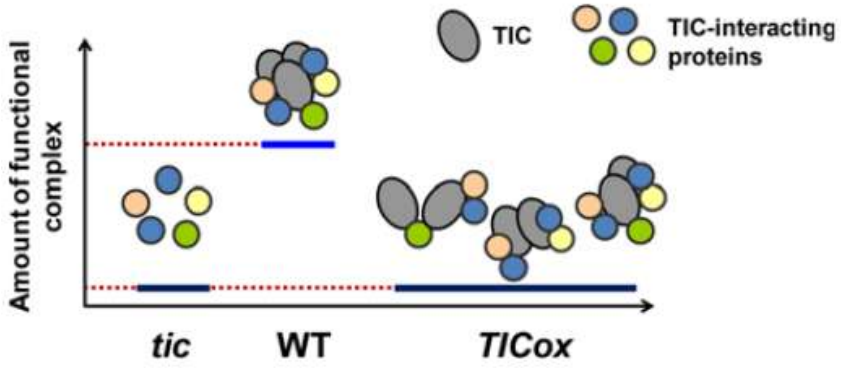


Figure 3.1.4-1. Titration hypothesis for a mode of biochemical activity of TIC. I propose that TIC forms heteromeric protein complexes and that correct stoichiometry of complex composition is critical for the homeostatic biochemical action of TIC.

3.2 TIME FOR COFFEE negatively regulates drought stress-responsive in ABA dependence single pathway of Arabidopsis

3.2.1 TIC maybe is an important ABA depended factor in drought stress responsible pathway

In spite of its functional importance on drought physiological responses and circadian clock regulations, the connection between drought stress and the circadian clock has not been defined. The *tic*-2 mutants displayed a phenotype of drought stress tolerance and short circadian period, but it is still unknown of functional regulation for TIC in these pathways [64,116]. In a previous study, TIC was suggested to be involved in protein degradation process, as depletion of its binding partner MYC2 was promoted by TIC [116].

Integration of external signals by this clock ensures metabolic homeostasis and ultimately enhances fitness. TIME FOR COFFEE (TIC) is known to be associated to the circadian clock, being required to maintain rhythmic period and amplitude, and to regulate clock-driven physiological responses. The molecular function of TIC has so far only been studied with loss-of-function mutants in Colombia background. The

biochemical activity of TIC remains elusive, but the condition of rhythmic period in TIC overexpression lines is still unknown. Previous research of rhythmic period and amplitude on tic mutant is only in Ws background, and the condition in Colombia background is still unknown.

Comparisons of circadian and ABA-responsive transcriptional profiles show that the genome-wide fraction of ABA-related genes controlled by the clock is around 40%, a significant percentage that is in agreement with previous studies [4] and with the notion that ABA signaling is under circadian control. The connection of the circadian clock with ABA pathways was also proven in studies showing the overlap between the ABA-related signaling molecule cyclic adenosine diphosphate ribose (cADPR) and the circadian transcriptome [42] and in studies in which a significant proportion of ABA-responsive genes was found to display diurnal oscillation [49]. Together, these data imply that the ABA pathway, if considered as a clock output, should be altered when the circadian clock is not properly functioning [12].

The Legnaioli's studies (2009) showed that ABAR is mis-regulated in TOC1-ox and TOC1 RNAi plants. In this publication, the studies with ABAR RNAi plants were consistent with the phenotypes reported in Arabidopsis, which are also in agreement with the presence of ABA-related motifs in the ABAR promoter and with the inclusion of ABAR as a gene regulated by ABA. The result also showed a significant hyposensitivity in ABAR RNAi plants to the ABA-mediated stomata closure, altered water-loss rates and decreased plant survival after dehydration. Furthermore, an inverse correlation between ABAR mRNA abundance and water-loss rates was observed, which highlights the important function of ABAR in the regulation of this plant response. ABAR function was also reinforced by genetic studies in which the TOC1 RNAi phenotypes were completely reverted by the ABAR RNAi construct, which in addition to providing clues about TOC1 and ABAR genetic interaction, also assigns a function for ABAR in the regulation of plant responses to drought [48].

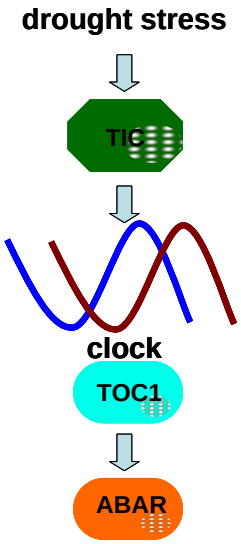


Figure 3.2.1-3 The hypothesis for drought and ABA response function of TIC, the TIC could be a acceptor of drought stress via to circadian clock, and the clock adjust the expression of core TOC1 which is the regulator for ABA and drought response gene ABAR

Here, I want to uncover ABA-related phenotypes for TIC loss-of-function lines and confirm the Ws background *tic*-1 mutant drought tolerance phenotype. These studies clarify the connection of drought stress response and the circadian clock as well as ABAR in the ABA signaling pathway. Current studies showed that TOC1 is induced by ABA, it is gated by the clock and determines the timing of TOC1 binding to the ABAR promoter. This study suggested that proper timing of this feedback loop is important for ABA-mediated changes in gene expression and plant responses to drought conditions. In the *tic* mutant the circadian clock genes are altered by shorter period that indicated TOC1 expression in tic mutant also changed. Taken together, we suspected that TIC is a connector between circadian clock and plant responses to drought, moreover, TIC could be a important infector on the input pathway from abiotic stress to circadian clock (Figure 3.2.1-3).

3.2.2 TIC is a connector between circadian clock and drought responses in Arabidopsis.

In previous reports the *tic*-2 (Columbia background) mutant response to drought stress was tested. For this, 3-week-old plants were subjected to a constant soil water

capacity (65, 30, 15, and 0%) for an additional 2 weeks. The growth and fitness of *tic*-2 was almost unaffected under any of these water-limited conditions, whereas Col plants suffered severely from drought at below 15% of soil-water capacity (tic2).

To investigating the molecular and biochemical function of TIC and complete the study of *tic* mutants' function, it is necessary to test *tic*-1 (Ws background) mutant response to drought stress. Thus, we improved the experiment method, growing the plants on the soil for 2 weeks and stop water until most wild type plants are dead. Thereafter we resumed watering plants for one week. Plants still living were then counted to make the calculation of survival.

I tested if *tic*-1 caused altered drought tolerance. Since *tic*-1 mutant lines expressed no functional TIC mRNA, and displayed circadian period phenotypes, we performed this analysis only with *tic*-1 and Ws wild type. Plants were germinated under 16 h light / 8 h dark (16L/8D) conditions on soil and started the drought stress treatment experiment as previously introduced. The result showed that *tic*-1 plants displayed a significantly higher drought tolerance; the tic1 mutant plants displayed a 35.83% survival rate in average of three replicate experiments, whereas it was 6.67% survival rate in average of three replicate experiments in the wild type (P-value: 0.029) (Figure. 3.2.2-1 A).

Thus, I next tested the TOC1 expression of *tic*-1 mutants to demonstrate if TIC causes altered TOC1 expression. Since *tic*1 lacks TIC function, and displayed largely identical phenotypes, we performed this analysis only with *tic*-1 mutants compared with Ws wild type. To monitor promoter activity of the circadian-clock evening-expressed and drought induced gene TOC1, we introduced a construct with the TOC1 promoter linked with luciferase (TOC1::LUC) into *tic*1 mutants and Ws wild type, and performed a luciferase reporter assay.

Plants were germinated and grown under 12 h light / 12 h dark (12L/12D) conditions for 8 days, then transferred into constant red and blue light (R+B) conditions. The rhythmic luciferase activity was monitored over 4 days under constant

light conditions. The TOC1 promoter periodicity and expression was analyzed. *tic*-1 plants displayed a significantly shortened period under constant R+B conditions. The *tic*-1 plants showed a 24.54 h±0.18 h (±SEM) period, whereas it was 28.96 h±0.17 h (±SEM) in the wild type (P value: 3.24E-22) (Figure 2.2.2-1 A, B, and C). The biological rhythm of TICox plants was well maintained without causing premature dampening of the oscillation (Figure 3.2.2-1 B). Furthermore, the rhythm of each individual plant was robust, as all plants displayed a relative amplitude error (RAE) below 0.6 (Figure 3.2.2-1 C). This result showed that *tic*-1 has a short period phenotype, whose rhythmic period was shortened [11-12].

To examine the effect of elevated TIC on the expression of drought stress response in ABA dependent pathway, I determined the transcript accumulation of various ABA and drought stress response genes. For this, plants were entrained under 12L/12D conditions for 7 days, then released into constant white light (LL) conditions for an additional 1 day. Plants were harvested at 8:00 morning and 20:00 evening or with 4 h intervals under LL, and total RNA was extracted.

To investigate the molecular and physiological function of TIC in drought stress response and ABA signal response, we examined the RNA accumulation of a gene which encoded ABA's synthesis key enzyme NCED3 under LL at 8:00 morning (ZT0) and 20:00 (ZT12) evening. The result showed that the *NCED3*'s has higher expression levels in the morning compared evening; this is consistent with current reports, which show that ABA has higher accumulation in the day than at night. The *tic*1 mutants were expressed to similar mean levels in the wild type both in the ZT0 and ZT12 time points (Figure. 3.2.2-2). This result indicated that the accumulation of ABA synthesis is not altered significantly in *tic*-1 mutants compared with wild type.

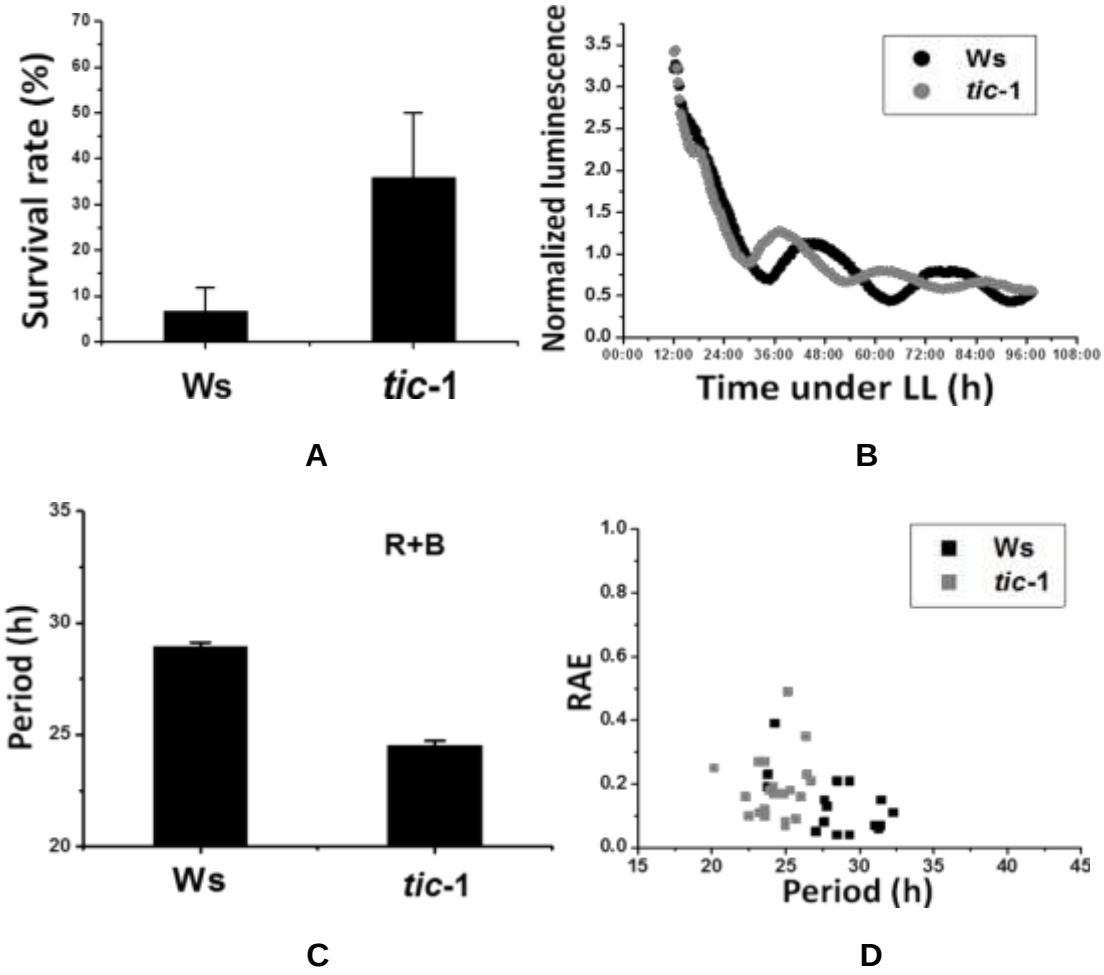


Figure 3.2.2-1 A tic-1 mutants displayed significantly drought tolerance phenotype compared with Ws wild type. The figure showed Ws wild type and tic-1survival rate under 1 week rewater after 2 weeks drought stress treatment. B-D *tic*-1 mutants displayed significantly shorter circadian period of TOC1 compared to Ws Wild type which harboring TOC1::LUC construct were entrained under 12 h light / 12 h dark conditions for 8 days, and transferred into constant light.

B. Normalized bioluminescence of TOC1::LUC

C. Period versus light conditions and ecotypes.

D. Period versus relative amplitude error (RAE) of individual plants.

The previous study has found that TOC1 binds to the promoter of the ABA-related gene (ABAR/CHLH/GUN5) and controls its circadian expression. TOC1 is in turn acutely induced by ABA and this induction advances the phase of TOC1 binding and modulates ABAR circadian expression (TOC1). On the other hand, our lab has performed real-time PCR on RNA extracted from replicate time points from *tic*-1 seedlings grown under 12-h-light/12-h-dark cycles. Through this assay, we found no major changes in mean expression levels of TOC1 as found in *tic*1

compared with wild type. In *tic*-1 mutants, TOC1 expression level is higher than wild type in the morning and lower in the evening, and the peak-to-peak and trough-to-trough distances of expressed genes were decreased [48]. These results are consistent with my luciferase reporter-assay data in Figure 3.2.2-1. Collectively, these results indicated that peak TOC1 expression in *tic*1 mutants was earlier appeared than wild type.

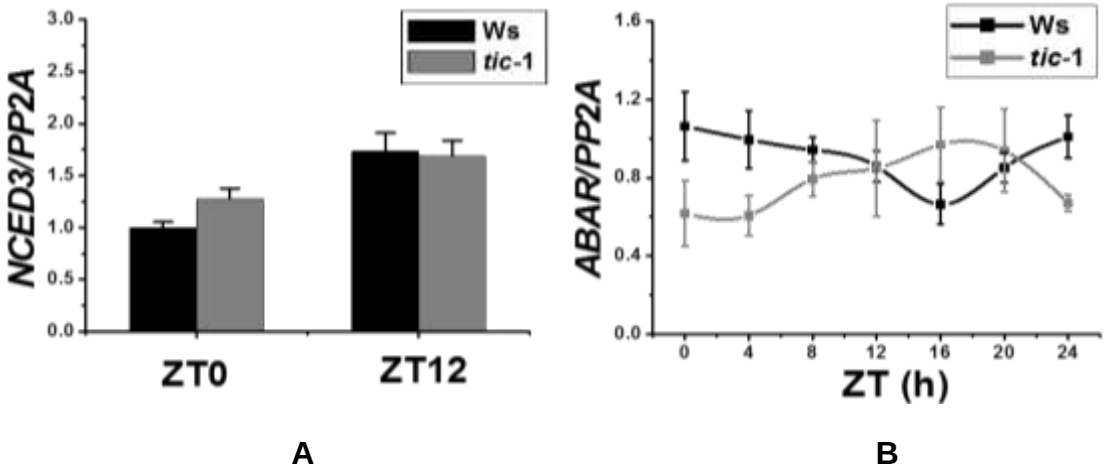


Figure 3.2.2-2 A. tic-1 mutants displayed similar NCED3 expression compared with Ws wild type both in morning (ZT0) and evening (ZT12). Ws wild type and tic-1 were germinated on MS1 plates, and grown under 12/12 light for 1 week. B. *tic*-1 mutants altered ABAR expression compared with Ws wild type during the daytime and nighttime. Ws wild type and tic-1 were germinated on MS1 plates, and grown under 12L/12D light condition for 1 week.

To gain insights into the mechanisms of *tic*-1 function in drought stress response, it is important to investigate how TIC regulates the ABA signal pathway in plants. Thus, I examined the RNA accumulation of an ABA hypersensitivity gene *ABAR* with 4 h intervals under LL light condition for 24 hours. (The primers of *ABAR* were adopted from Legnaioli et al 2009[48]). The result showed that the expression conditions of *ABAR* are different in independent ZT time during 24 hours. The expression level of *ABAR* was higher in the daytime and lower in the nighttime in the wild type, but showed the opposite pattern in *tic*-1 mutants, with higher expression levels appearing in the evening (Figure 3.2.2-2).

Due to higher accumulation of ABA at night time, the ABA hypersensitivity gene *ABAR* had lower expression in nighttime and expected low enrichment level of response to the ABA signal. *tic*-1 altered *ABAR* expression condition, and thus, increased drought tolerance.

3.2.3 TIC could regulate plant drought stress responses by phosphorylation modification

TIC causes a negative regulation of drought stress tolerance in plants, and is involved in the ABA dependent pathway. Despite the functional importance on the alteration for drought tolerance physiological responses of TIC, the mechanism of biochemical and molecular function of TIC in the response to drought stress has not been defined. Study of the structure and sequence of TIC showed a protein composed of 1555 amino acids and has no similar structure with other protein. Thus, no known functional motif can be found in its primary structure [12].

To investigate the molecular and biochemical functions of TIC in drought stress response, I measured the expression of *TIC* genes in non-stressed Ws wild type compared with those drought stress induced. The drought stress treatment method was replicated to that of the plants survival rate experiment as before, but without resuming water treatment after drought, with plants harvested after drought. After RNA extraction, transcription, and RT-PCR performed, the result showed no significant difference of *TIC* between drought stress and non-stress conditions (Figure 3.2.3-1). The result of drought treatment lines' displayed levels didn't increase through the treatment. This indicates that the drought stress didn't induce any transcriptional changes of *TIC*.

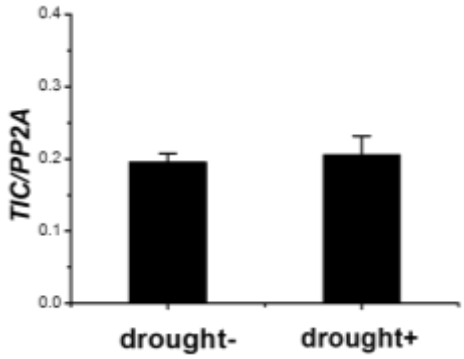
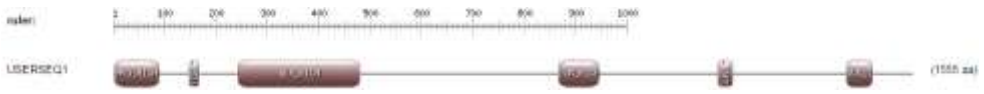


Figure 3.2.3-1 The TIC gene expression in Ws wild type plants under drought treatment. The Ws wild type seedlings were germinated on soil, and growing under long day (16L/8D) light condition for 2 week. The drought + line was under 1 week no water treatment.

As the previous results showed that unfunction *TIC* increased drought stress tolerance in plants, but the drought stress didn't significantly induce *TIC* to change its transcription level. In a previous study, TIC protein accumulation was in the nucleus and only reported to negatively regulate the tolerance of plant pathogens stress by protein interaction with a binding partner MYC2. That result showed that TIC was suggested to be involved in protein degradation process. Except this, no other publication reported protein level function of TIC. To investigate however TIC may function on protein level in plants, I utilized bioinformatic analysis and prediction of structure and function of protein sequences., Using Prosite Expasy (http://prosite.expasy.org/), the data base of protein domains, families and functional sites as a tool to analyze the TIC protein sequence, I tried to find some potential functional domains. The analysis result showed that there is a Serine rich domain near the N terminal part of TIC, from 244 to 482 amino-acid. This was indicated that TIC could be a potential kinase substrate, and also suggested that the mechanism for TIC's regulation could be realized by its phosphorylation modification (Figure 3.2.3-2).

Due to a large numbers of Serine on the Serine rich domain of TIC, it is difficult to try every Serine on that domain. Thus, it is necessary to find out some potential phosphorylation sites. I used the Internet service Kinasephos (http://kinasephos.mbc.nctu.edu.tw/index.php) to see if there are any potential phosphorylation sites on TIC full length. The result showed that there are 88 potential

phosphorylation sites on TIC full length and 38 of them were on the Serine rich domain as previously analysis (Figure 3.2.3-3).



A

MDRNREARRVPMAAAGNGLSRRRHRAGSFRDSPEEEGPVELPEAARLRDRGGSNKKDRDRERDRDR
ERERERDRERDRLNSRSKRRRGERLMMVHGNLDDGGDDSSEESVNDDEEYDDGGVGPPSSLKMLPP
TSNNISAASFSSSLSNHHNGGSGNLHHHHHSHNNNHQRKNNFPPTKVFRSSPSPAPVSPLVSTWKA
ADEMIGVSVPRKARSACTKRPHESWASSTTGGGVFASGEQIHRQISSTSPANRVSPASILASPSPP
APTSPSSSSSISVRKKLPSGTKQKPLPPKSSSSKLSSPVAVQDEIEIEIAEVLYGMMEMPSTSKQEA
AGNDLTEAAKSTVEVKSRVSSPISNFQTLPQSSITLAANSSSSNVSAIAPKRKKPRHVKYEDDNSS
RVTTIKSEAEAPSKSQVPFSNQLKSSGSGEGNSSVLDSIIPLTRESNASLDSEKKENNLSKDETIL
PKVESSSGFRSDGEGAKSSSPEKEKFEIDLMAPFPVRSSSERGGEMMECVAAEAKPKVTEVETEAK
PLLKEDRSDPAIHDSQEKKRPRMVAEAEHHKFERNCELKLDLDKSDHVGLVNKHHVQKPPPQQQLS
VPDKTAQASHLPLHMSMPGWPGGLPTMGYMAPTQGVVPTDTSSLSAAAMQPPPHLLFNQPRPKRCA
THCYIARNIQSHQQFTKMNPFWPAAAGSAPMYGTKACNLSLMPPTELQGSVLGRSSNPVQDKNSQS
TSKSSETAQRNQLMLQQALPPGAANSILHGPTFIFPLGQQPHAAATIAAASVRPPNSGITSSGPTA
TSTSMNGSASATPAGAPTMSFSYPAMPGNETQYLAILQNNGYPFPVPAHVGAQPAYRGAPGQPMPF
FNGSFYSSQMIQPPHHQPQKQHQQQLTGQMLQSHAPNNQNGSASTGSSAAQKHLQNQQLRPPINHG
NSQGFPTHKVQSQPLNFQQRQQPRENATQHSETVGEDSPSTADSRGSRSNVAYGQNYGMQMQPTNL
GLMSSPAPGGGVVGSSSSHGEKKSQQQVSKAGVESFQSPGYAMTFATFNGANTAPTLNMSSIAQNH
AMFHSMPEAARQGYQMMAAQAAQQKMNYGASLEDGKSGSIGGAATANNTPEEQRKSGGGAIGKTSG
GNGGQSIAFSNKQDLADASVSAVTSGSIVDSSSRLLNLGSALPQSSGSLPTSHHQQLLQQQQQQHM
QRSQSQQPYTTMYLQKQQRYATSVAASAARTKGPVVSNGSGFFDHNMTTSPAGTTKFANANSGFPQ
NLVQSSSNQVQSQQWKNNSPRTTNTTQAQSPSMLSPSTSVAAASSLRNIPHKQQSRPQQSQISFAA
NSKPMTSGSPMQQVQGGTNHQAPSPPMLVGSPSTSSVSKNASGSPRTTASASSAANKGGQASTTTH
SASQPSKNLQPASAASSAGGRNNGPSVLGNPTTSSGSKSQQQQQLPKHGLQPQAQLFFSNPYMQAQ
HQHQQQQITISPSGGYYIQRHQQQSGSAPAVPVTGAVTATSDPAKAIAAASAANNMKGGGGMGKTQ
QHQLGPPGFTNVHAVSSAVQVKPVDQKQQAACAGETK

B

Figure 3.2.3-2 Analysis result of TIC protein sequence in Prosite Expasy.
A. Different potential domains' position of TIC, the third brown band is potential Ser rich domain.
B. Full sequence of TIC, the yellow grounding part is potential Ser rich domain.

To uncover the biological consequence of TIC phosphorylation, and identify several potential phosphorylation sites on TIC, I made a homologous sequence alignment with 27 different other plant species, assertain conserved sequences shared between different species. The result showed there are several, conserved Serine on the Serine rich domain, which indicated that these Serine could be the potential phosphorylation sites (Figure 3.2.3-4).

In order to verify the function of these potential phosphorylation sites, I chose some of these different sites, used the mutagenesis technique to mutate these encoded phosphor-acceptor sites in TIC and then introduced these site-directed mutation constructions into the *tic*-1 mutant background to examine the restoration capacity and use the TIC full length without any mutagenesis site as control. If the serine is a

phosphorylation site, the transgenic plants would show some phenotype.

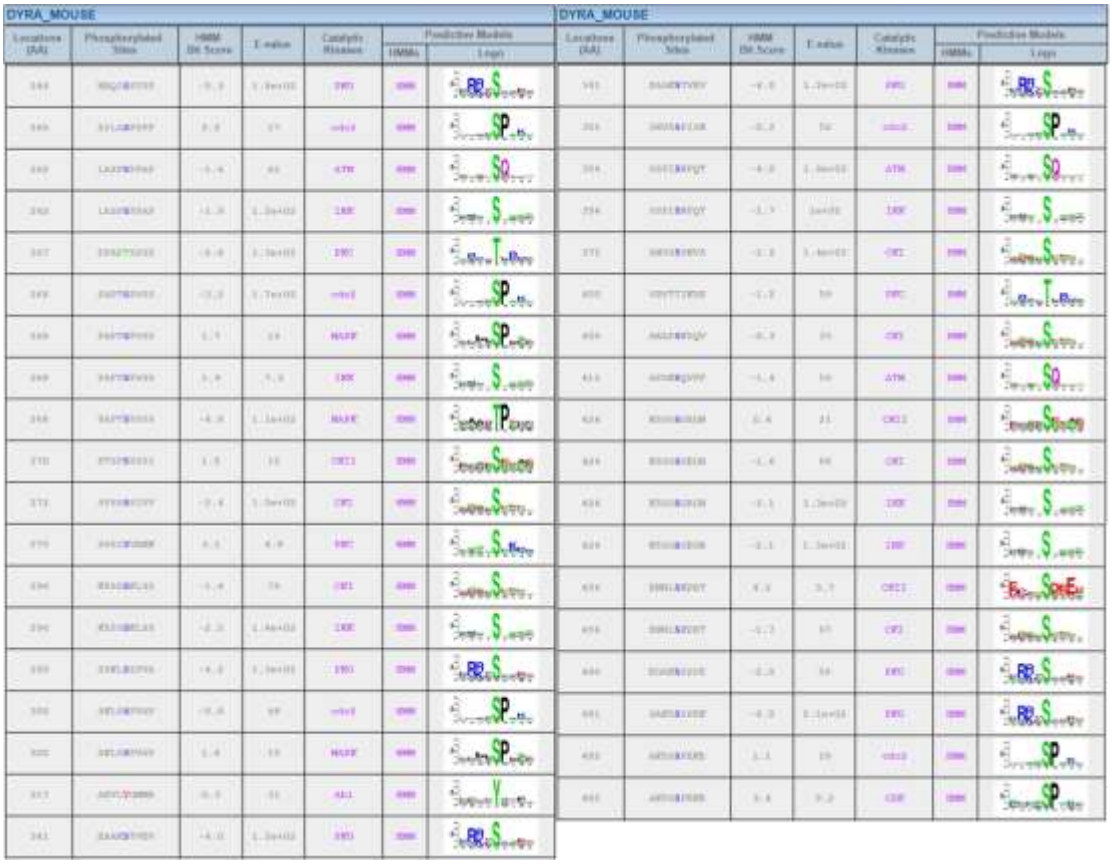


Figure 3.2.3-3 Analysis result of TIC Ser rich domain (amino-acid 244 to 482) in Kinasephos. 38 potential phosphorylation sites was fund in the scanning, include No.351 serine.

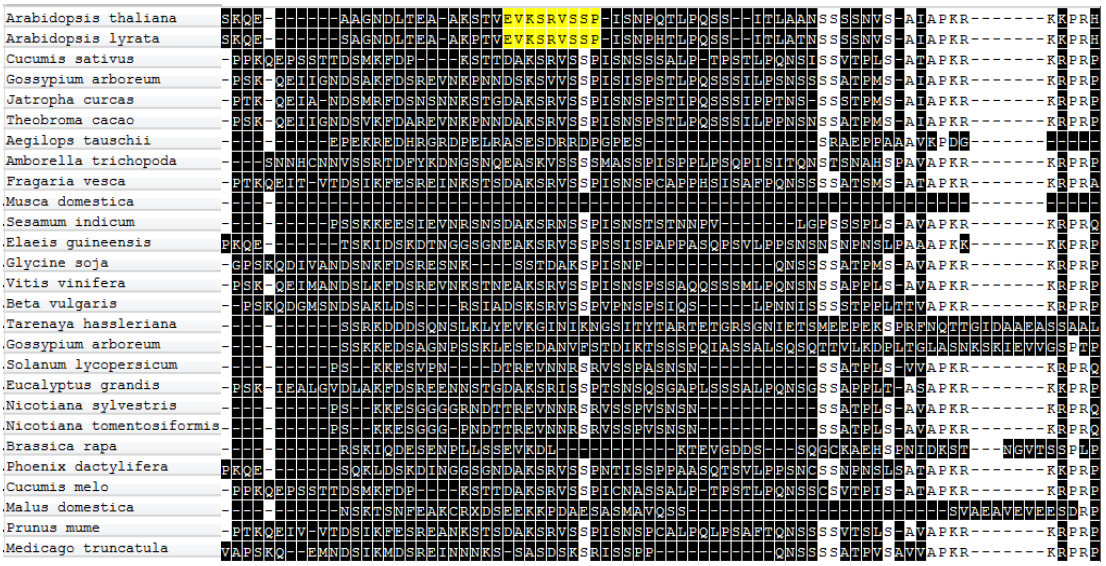


Figure 3.2.3-4 The homologous sequence alignment with 27 different other plant species and Arabidopsis thaliana of TIC, the yellow background part is the conservative sequence fragment, the last serine of this fragment is No.351 serine and appeared in 21 different plant species.

For construct point mutations, I used the vector with full length TIC sequence and designed the primer, which was used to do the mutagenesis with Quik-change method. The primer included 25 nucleic acid bases of double sides of the mutation place, and the mutations place included the mutation bases. Next, the PCR program followed as the protocol in materials and methods part in this thesis.

To test the *in vivo* significance of the phosphorylation activities that were previously quantified for drought response function of TIC in vitro, we stably transformed Arabidopsis plants with transgenes in which the 35S promoter drives expression of wild-type TIC or with Serine-to-Alanine substitutions in the amino acid sequence of the TIC protein.

Finally, I was interested in one Serine-to-Alanine substitution, which was No.351 (Figure 3.2.3-5) Serine mutant to Alanine named as S351A. The S351A substitution is a site mutation of a potential phosphorylation site. This site was found both in the Serine rich domain, which from the Prosite Expasy analysis and potential phosphorylation site from Kinasephos analysis. Changing residues that are homologous to Serine 351 to Ala in TIC un-functionally mutants *tic*-1 in vitro. I transformed Arabidopsis plants that are heterozygous for *tic*-1, a T-DNA insertion allele that is a null, these lines were named as *tic*-1-35S::TIC-S351A (short writing in *tic*-1-S351A), and homozygous for a T-DNA insertion allele of TIC with 35S promoter as a control. The control lines were named as *tic*-1-35S::TIC (short writing in *tic*-1- TIC) (Figure 3.2.3-6). Next, I identified stably transformed Arabidopsis plants that contain single *tic*-1 mutant transgenes by scoring antibiotic resistance in segregating populations. I then isolated lines that are homozygous for the individual TIC-expressing transgenes. Firstly, the transgenic T1 lines displayed 33% positive seedlings in the resistance selection, next, selected T2 lines displayed 100% positive seedlings in the resistance selection and used PCR to do genotyping. The genotyping PCR method was performed as the protocol in materials and methods part in this thesis (Figure 3.2.3-7).

After finding homozygous lines of *tic*-1-S351A and *tic*-1-TIC control, I performed the drought treatment tests for these two lines and the *tic*-1 mutant and Ws wild type together. This experiment was used to confirm this site directly mutation of TIC's phenotype is like *tic*-1 mutant or wild type, and also detect the *tic*1-TIC control could make phenotypic covering of TIC.

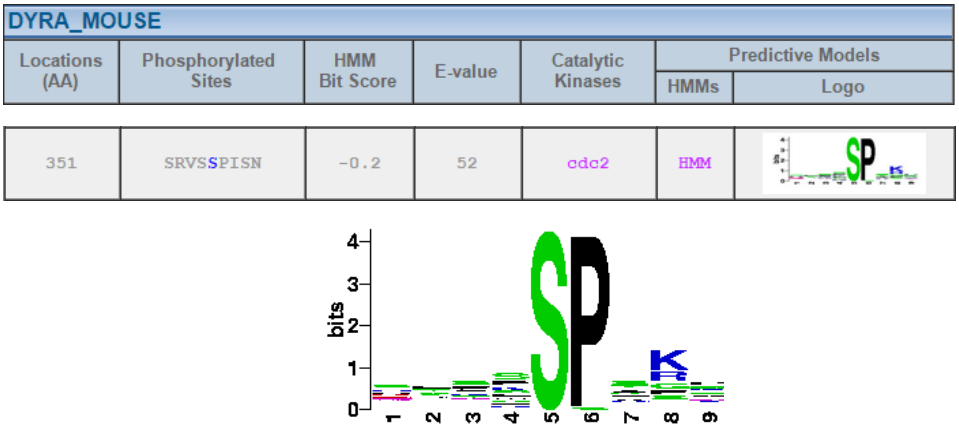


Figure 3.2.3-5 Analysis result of No.351 Serine in Kinasephos.

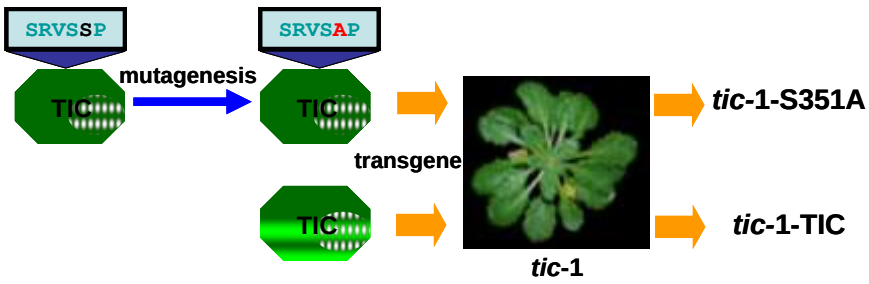


Figure 3.2.3-6 Protocol of making Serine-to-Alanine directly site mutant lines and TIC full lengthe control lines.

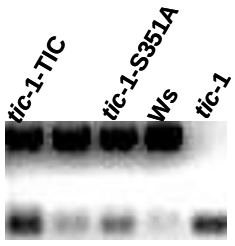


Figure 3.2.3-7 Genotyping results for Ws wild type, *tic*-1, *tic*-1-S351A and *tic*-1-TIC, gnomic DNA was extracted from 1 week old seedlings. The PCR production with EcoR I HF treatment for 3 hours, the tic-1 displayed smaller size band compare with wild type, *tic*-1-S351A and *tic*-1-TIC had tic-1 back ground but expressing TIC sequence, thus, they displayed both bigger band as wild type and smaller band as *tic*-1 mutants.

The result of the drought treatment showed that the *tic*-1-S351A line plants displayed 31.67%±0.72 (±SD) survival rate under the drought stress; significantly higher than Ws wild type (6.67%±0.52 (±SD) P-value: 0.0052) and this tolerance phenotype was similar as *tic*1. Together, the *tic*1-TIC control lines displayed 13.33%±0.11 (±SD) survival rate, and it didn't show significant drought tolerance phenotype compare with *tic*-1 and significantly lower than *tic*-1-S351A line (P-value: 0.041), but still showed a no appreciable higher survival rate than Ws wild type (P-value: 0.21) (Figure 3.2.3-8).

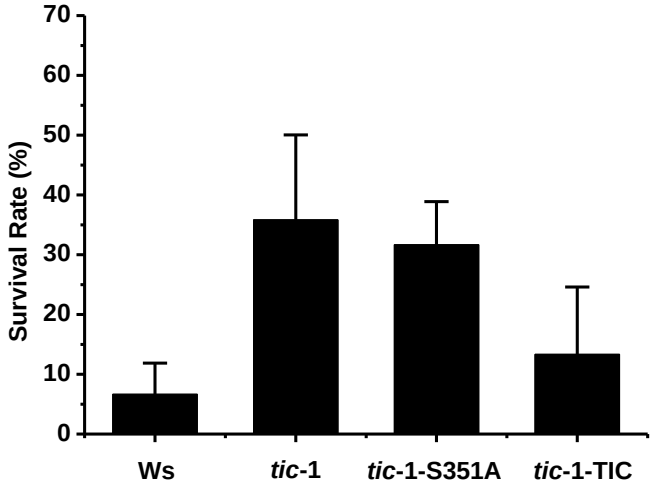


Figure 3.2.3-8 *tic*1-S351A line displayed similar drought tolerance phenotype as *tic*-1 mutants, and significantly more drought tolerance phenotype compared with Ws wild type and *tic*-1-TIC control. The figure showed Ws wild type and tic-1survival rate under the 1 week rewater after 2 weeks drought stress by stop water.

3.2.4 Other phenotype investigations of *tic* mutants.

3.2.4.1 *tic*-1 mutants displayed late blotting time phenotype.

It is becoming increasingly evident that TIC also controls development and flowering time in plants; however, these roles are not well established and the underlying molecular mechanisms [11] are not known. The observation that inhibition of development and flowering time in *tic* mutants of Colombia wild type provided the first evidence for a role of TIC in development and flowering time, but the condition

in Ws wild type is still unknown. As the previous result indicated that the mechanism of TIC function is based on the protein modification or degradation, but the influence of the altering of TIC transcription model is still unknown.

To examine the effect of expression of TIC control in development and flowering time in plants, I next tested if *tic*1 mutant caused altered flowering time and leaf growth time. Since the *tic*-1-TIC and *tic*-1-S351A transgenic lines were made and displayed largely identical phenotypes, I performed flowering time and leaf growth analysis with *tic*1 line and Ws wild type. To monitor the flowering time, I counted the different the leaves numbers at the flowering time of every individual plant lines include *tic*-1-TIC, *tic*-1-S351A, *tic*-1 and Ws wild type under long day conditions. The detail protocol was followed the method in materials and methods part in this thesis.

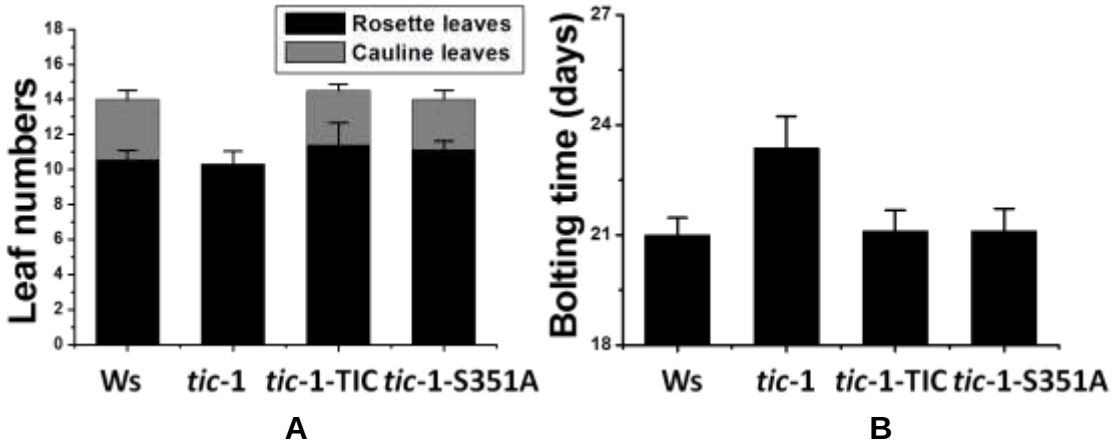


Figure 3.2.4-1 Flowering time and bolting time analysis results of WS, *tic*-1, *tic*-1-TIC, and *tic*-1-S351A lines.

A. *tic*-1 didn't display late flowering phenotype compare with WS, *tic*-1-TIC, and *tic*-1-S351A.

B. *tic*-1 displayed late bolting time phenotype compare with WS, *tic*-1-TIC, and *tic*-1-S351A.

When grown in soil, the *tic*-1 plants didn't display late flowering phenotype (having more rosette leaves when they were flowering) compared to the wild type; under the long day condition of 16h daytime and 8h nighttime, the *tic*-1 plants produced 10.33±0.71 (±SD) rosette leaves before flowering, and the Ws wild type produced 10.55±0.53 (±SD) (P-value: 0.23), this result was unlike the *tic*-2 delayed longer time in transition from vegetative to reproductive phase under inductive

long-day conditions (Figure 2.2.4-1 A). However it is clearly shown that when *tic*-1 plants started to flower (0 cauline leaves), the wild type plants had already produced 3.4±0.53 (±SD) cauline leaves; this indicated that the *tic*-1 mutants showed later bolting date compared to the wild type. Taking this further, I counted the bolting date of these plant lines, the *tic*-1 mutants were bolting at 23.38±0.86 (±SD) days, and it was significantly later than 21±0.47 (±SD) days of wild type (P-value: 2.62E-5) (Figure 2.2.4-1 B).

Under the same growing condition as above, the *tic*-1-TIC line plants display slightly late flowering phenotype compared to the wild type. At the flowering time, the *tic*-1-TIC line produced 11.38±1.30 (±SD) rosette leaves and the Ws wild type produced 10.55±0.53 (±SD) rosette leaves (P-value: 0.065). In the bolting date analysis of these line plants, the *tic*-1-TIC plants bolting date was at 21.13±0.60 (±SD) days, it displayed similar bolting date compare with Ws wild type (21.0±0.47 (±SD)) (Figure 2.2.4-1A B).

In the same experiment with *tic*-1-S351A line plants, they produced 11.11±0.53 (±SD) rosette leaves in the flowering time analysis, and the bolting date was at 21.11±0.57 (±SD) in the bolting date experiment, these two results of *tic*-1-S351A line plants were both displayed similar phenotype as *tic*-1-TIC line plants (Figure 2.2.4-1).

3.2.4.2 *tic*-1 mutants are defected of increasing of anthocyanins under ABA stress condition

In current reports, the accumulation of anthocyanins in different plant tissues is considered as a response for biotic or abiotic stress. Given the induction of anthocyanins by osmotic stress, it is not surprising to find that plant tissues containing anthocyanins are often resistant to drought stress. From observations made of *tic*-1 mutants and the Ws wild type in the stress treatment experiment, the Ws wild type showed a purple color on the leaves under drought stress but the tic-1 mutants had no significantly color change. It is noteworthy that the *tic*-1 mutants and Ws wild type

plants changes of anthocyanins in stress conditions.

To examine the effect of TIC in the anthocyanins accumulation under the stress, I prepared MS1 medium plates with 5μM ABA and transformed 2 week old seedlings on them, and grew similar numbers of seedlings on normal plates as a control. The stress treatment was as described in the protocol in materials and methods part in this thesis. When grown on normal MS1 plates, the *tic*-1 mutants displayed a similar phenotype compared with growing in soil. Thus, the Ws wild type presented purple stain on rosette leaves under the ABA treatment, but *tic*-1 did not display this phenotype under the ABA stress.

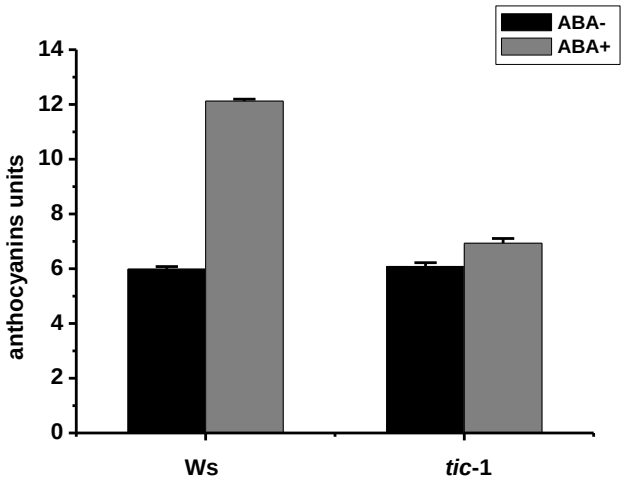


Figure 3.2.4-2 The Ws wild type and *tic*-1 mutants were germinated on MS1 plates and grown for 2 weeks, the ABA treatment lines were treated with 5μM ABA (solved in EtOH), and the control lines were grown on MS1 with EtOH plates. In the ABA treatment condition, the anthocyanins was increase to 2 times compared without ABA treatment in Ws wild type, but *tic*-1 mutants didn't show significantly different anthocyanins content with ABA treatment.

Furthermore I detected anthocyanins of every line under the ABA stress and normal condition, and counted the content changing between two conditions. The result showed that *tic*-1 3 week old seedlings displayed 6.08±0.14 (±SD) anthocyanins unite in the normal growing condition on MS1 medium plates, and the same old seedlings which was under 1 week normal condition and 1 week ABA stress treatment displayed 6.93±0.17 (±SD) and increased 0.85±0.15 (±SD) under the

treatment, (Figure 3.2.4-2) it was significantly lower compared with Ws wild type (5.99±0.09 (±SD) in normal condition, 12.12±0.07 (±SD) in ABA treatment, 6.14±0.07 increased, P-value: 3.86E-8 vs *tic*-1) (Figure 3.2.4-2). The result showed that Ws wild type significantly increased accumulation of anthocyanins under the ABA stress; *tic*-1 line plants displayed significantly defect in the increase of anthocyanins accumulation under the ABA stress treatment.

3.2.5 Discussion

TIC is an important regulator for clock activity in clock input pathway. Thus, the circadian period and amplitude was changed in *tic* mutants, and TIC protein also regulated clock-driven output responses for biological stress abiotic stress [12,64,116]. However, the exact function of stress responses of TIC has not been clearly defined. In this study, I generated *tic*-1, *tic*-1-TIC, and *tic*-1-S351A plants and characterized their circadian clock gene period, drought tolerance, growth and flowering and anthocyanins content phenotypes compared with Ws wild type.

In the result of drought stress test, I found that *tic*-1 plants displayed comparable drought stress tolerance phenotypes as *tic*-2 mutants, (Figure 3.2.2-1 A) the difference expression model of drought response genes *TOC1* and *ABAR* indicated that TIC was an important connector between the clock and drought stress response [48]. As the investigation of the normal expression model of *ABAR* in Ws wild type and *tic*-1 mutants, the *ABAR* displayed a different transcription level during daytime and nighttime and the high expression was observed in daytime and low expression in the nighttime. *tic*-1 mutants was displayed the opposite expression condition; the high expression in the nighttime and low expression in the daytime (Figure 3.2.2-2). The ABA hypersensitivity gene *ABAR* had lower expression in nighttime and an expected low enrichment level of response to the ABA signal and drought stress of plants. Since TIC lacks function, the period for gene transcription of *TOC1* was shorter compare with wild type (Figure 3.2.2-1 B-D). In previous studies, TOC1 inhibited ABAR in transcription level [48], the shorter period of *TOC1* caused the delay of the

inhibition for *ABAR*. Due to the ABA had higher accumulation in plants at nighttime, thus, in this condition, *ABAR* had higher expression in nighttime and expected stronger enrichment level of response to the ABA signal and drought stress of plants than wild type. Thus, these results indicated that TIC is an important connector between the stress response and circadian clock, through timing control, built a TIC-TOC1-ABAR regulation signal pathway, and played an important role in the ABA response. Subsequently, the role of TIC is that of a negative regulation in drought stress response through the circadian clock component TOC1 in ABA dependent signal pathway in plants (Figure 3.2.5-1).

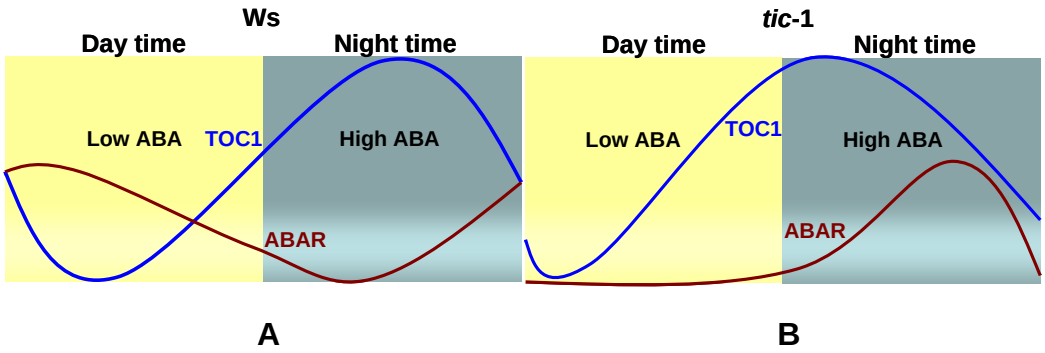


Figure 3.2.5-1 *TOC1* and *ABAR* expressing condition in Ws wild type and tic-1
A. *ABAR* in Ws wild type displayed high expressing pick in morning and low pick in evening.
B. *ABAR* in *tic*-1 mutants displayed high expressing pick in evening and low pick in morning.

The serine to alanine site mutant *tic*-1-S351A line displayed a similar drought tolerance phenotype compared with *tic*-1 mutants, and the *tic*-1-TIC line didn't show a significant drought tolerance phenotype (Figure 3.2.3-8). This result indicated that this defection of potential phosphorylation site was affected by TIC function of drought response. Furthermore, it could indicate TIC function with phosphorylation-dephosphorylation modification. In additional, due to the *tic*-1-TIC, *tic*-1-S351A lines were transformed by the construct of 35S::GFP-TIC with 35S promoter but not TIC native promoter, the result also showed that 35S promoter didn't change drought stress response function of TIC (Figure 3.2.5-2).

I noted that disparate hormone pathways converge in flowering-time regulation [117-118]. The *tic*-2 mutants consistently resembled each other in various phenotypic

aspects. With the investigation of *tic*-1 mutants, unlike *tic*-2 mutant, *tic*-1 plants didn't display a significant late flowering phenotype (Figure 3.2.4-1 A), but did display a late bolting time phenotype. This result indicated that *tic*-1 mutants could also display growth defect as similar as *tic*-2 (Figure 3.2.4-1 B-D). Considering the phenotypic resemblance of Ws wild type, *tic*-1-TIC, and *tic*-1-S351A in flowering time, this result was similar as the result of drought tolerance test and demonstrated that the 35S promoter didn't significantly affect the function in flowering time regulation of TIC. We hypothesize that TIC may form an important kinas substrate protein and function with phosphorylation-dephosphorylation modification, it is critical for the drought stress response but not for flowering time (Figure 3.2.5-2). In conclusion, lack of TIC may inhibit the growth of rosette leaves under long-day conditions, but didn't delay the time of transition from vegetative to reproductive phase under long-day conditions.

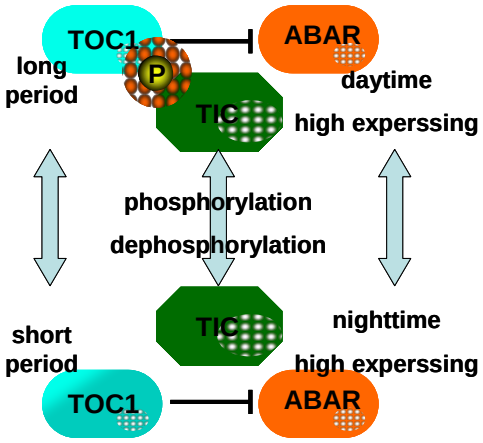


Figure 2.2.5-2 The results indicated that the connector function drought stress response in ABA depended pathway and circadian clock via phosphorylation-dephosphorylation modification on TIC.

In a previous study, it was shown that anthocyanin accumulation is an important response when plants are under stress. I show that anthocyanin accumulation was increased under ABA stress in Ws wild type. In *tic*-1 mutant of the same genetic background, they displayed defects of increasing anthocyanin accumulation under ABA stress (Figure 3.2.4-2). In further studies of this thesis, the *tic*-1 mutants displayed drought stress tolerance phenotypes and involved ABA dependent pathway. All these results indicated that ABA also could be a signal of stress to anthocyanins

synthetic, and TIC loss-of-function plants defect in regulation from the stress to anthocyanins synthetic and stop the increasing of anthocyanins accumulation under the stress (Figure 3.2.5-3).

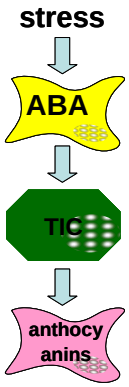


Figure 2.2.5-3 The results indicated that TIC regulated anthocyanins synthetic in the ABA stress, the anthocyanins will be up regulated with ABA stress condition in plants, TIC could be a regulator in this pathway, the anthocyanins didn't up regulated with ABA stress in *tic*-1 mutants.

3.3 AKIN10 interacts and phosphorylates TIC

3.3.1 TIC could be regulated on phosphorylation in circadian clock by AKIN10

Despite that both AKIN10 and TIC have important functional regulations on the circadian clock [12,119], the mechanism of regulation between AKIN10 and TIC has not been defined. In previous studies, TIC was confirmed as a circadian clock regulator. The *tic*-2 mutants displayed a phenotype of drought stress tolerance and short circadian period, but it is still unknown the functional regulation of TIC in these pathways. However, TIC is considered as a clock regulator not under circadian control. Ding et al. (2007) previously demonstrated that TIC mRNA and its protein levels did not display circadian oscillation during the day [12], and TIC effects on the circadian clock is time specific as noted by a clock arrest before dawn [11].

Consequently elucidating a mechanism, by which TIC acquires a time-specific

action on the circadian clock, in particular to clock entrainment, may lead to a better understating of TIC function. With this aim in mind, a biochemical approach was undertaken to begin to understand TIC function. I was interested in one of the main candidates from the yeast two hybrid screens which is protein kinase AKIN10 and this interaction was confirmed in yeast. AKIN10 was involved in stress responses, in particular to carbohydrate availability and energy signaling in the dark to light transitions [77].

Compared with the result of circadian period analysis of *AKIN10*, *tic*-2 and *tic*-2/*AKIN10* under β-estradiol induction, AKIN10 was overexpressed., A shorter period phenotype was observed on *tic*-2/*AKIN10* and *tic*-2 mutants but *AKIN10* showed longer period compared with wild type. Thus, we conclude AKIN10 is upstream of TIC in circadian clock, but the regulation mechanism is still unknown.

Conclusive the previous results, and based on the interaction between AKIN10 and TIC was confirmed, here I wanted to investigate if AKIN10 could phosphorylate TIC and map the phosphorylation sites of TIC if it is existed. This is important to clarify the role of interaction between kinase and circadian clock companion. Furthermore, TIC was known as an important regulator in the input pathway of circadian clock, it affects the core loop of TOC1 and CCA1/LHY before dawn. Thus, it is also could explain the mechanism of the energy transformation on the input pathway of circadian clock. In the *tic* mutant the circadian period was shorter whether AKIN10 was overexpressed or not, but longer circadian period phenotype was observed when AKIN10 was overexpressed in wild type background. Together, I strongly suspected that AKIN10 could phosphorylate TIC and this modification on TIC could be an important role for TIC in the input pathway of circadian clock (Figure 2.3.1-3).

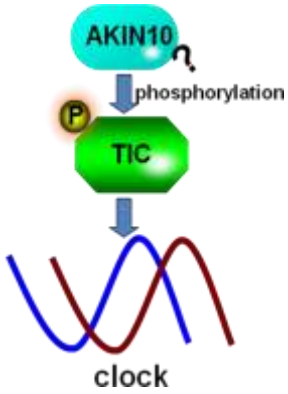


Figure 2.3.1-3 The hypothesis of the phosphorylation between AKIN10 and TIC. The circadian clock could be regulated by TIC via the phosphorylation modification from AKIN10

3.3.2 TIC is phosphorylated by AKIN10 in vitro

As previously reported, the interaction between TIC and AKIN10 is already proved in vitro; the TIC fragment 427 to 578 was shown to clearly bind with AKIN10. Despite the kinase identity of AKIN10, I tested if AKIN10 could phosphorylate TIC or not. Luminescence results of *AKIN10*, *tic*-2 and *tic*-2/*AKIN10* showed that AKIN10 could be TIC's upstream regulator of circadian clock. I made an AKIN10 and TIC fragment prokaryotic expression, thus, use the γ-32P ATP to test the phosphorylation activity of AKIN10-TIC fragment interaction.

To investigate whether TIC can serve as substrates for AKIN10, we performed *in vitro* interaction and phosphorylation assays. I used TIC constructs from Ding (unpublished) The construct includes a TIC fragment from 427 to 578, for mark this fragment and be different from the TIC full length. I named this fragment as TICzj.

The TICzj protein fragments were fused with glutathione S-transferase (GST) and expressed in Escherichia coli, respectively. The GST fusion proteins were purified by affinity chromatography using a Glutathione Sepharose 4B column. The AKIN10 (full length) constructs was provided by Dr. Mihály Horváth from Dr. Csaba Koncz lab in our department, the AKIN10 proteins were fused with Histidine-tag (His) and also expressed in *Escherichia coli.*

To examine the interaction between AKIN10 and TIC in vitro, I generated His-AKIN10 and GST-TICzj fusion proteins to be used in GST-pull down assays.

Thus, I performed an in vitro pull-down assay between recombinant His-AKIN10 and GST-TICzj. This showed that His- AKIN10 could directly interact GST-TICzj (Figure 2.3.2-1).

The *in vitro* protein phosphorylation was detected by autoradiography with γ-32P ATP. As the results show in Figure 3.3.2-1, when the autophosphorylated His-AKIN10 (lane 1), single purified GST-TICzj protein (lane 2), or the autophosphorylated His-AKIN10 together with GST-TICzj (lane 3) were incubated in the γ-32P ATP containing protein phosphorylation reaction mixture, only the GST-TICzj (lane 2) did not display any blot on the autoradiography. His-AKIN10 (lane 1) showed an autophosphorylated band, and His-AKIN10 together with GST-TICzj showed two bands on these two proteins position. This result confirmed that the GST-TICzj protein was phosphorylated by His-AKIN10.

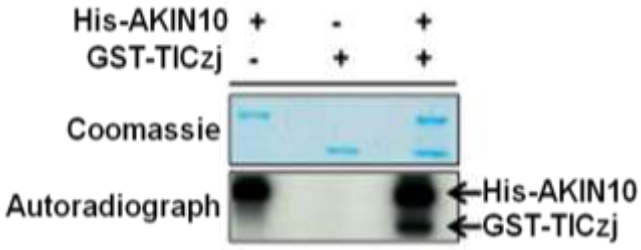


Figure 3.3.2-1 Upside figure was the pull-down result of His-AKIN10 and GST-TICzj, lane 1 and lane 2 was only 1μM AKIN10 and TICzj, lane 3 was both proteins with protein interaction buffer and purified with GST-beads, all the samples was on the PAGE gel and stained with coomassie blue. The bottom figure was the autoradiograh of kinase assay of His-AKIN10 and GST-TICzj, each lane used the same sample as pull-down experiment but lane 3 used kinase reaction buffer and 5μCi γ-32P ATP.

3.3.3 No.466 serine on TIC is the phosphorylation site for AKIN10 *in vitro*

The interaction and phosphorylation between AKIN10 and TIC result is important to demonstrate the mechanism of regulation between AKIN10 and TIC, but the TIC phosphorylation sites of AKIN10 are still unknown. To further understand this biochemical incident and explain the possible mechanism of regulation between

AKIN10 and TIC, it is worth mapping TIC phosphorylation sites for AKIN10. To do this, I decided to use mass spectrometry (MS).

As previous bioinformatic analysis of the TIC protein sequence, I have shown that No. 244 to 482 amino-acid on TIC protein sequence and the TICzj fragment is No. 427 to 578 amino-acid on TIC protein sequence. Thus, it consists a highly conserved and serine rich domain. To determine the TIC phosphorylation sites resulting from in vitro His-AKIN10 and GST-TICzj kinase assay reaction system, I purified both of His-AKIN10 and GST-TICzj protein and incubated them with ATP in the reaction system with kinase reaction buffer for over 2 hours. Thus, the completed transphosphorylation from autophosphorylated His-AKIN10 to GST-TICzj was finished. I used phosphorylation protein purified column to obtain the enrichment of phosphorylated protein in this reaction system. To use the mass spectrometry (MS) analysis, I used the trypsin digestion method to digesting the His-AKIN10 and GST-TICzj to small peptides.

After several attempts of MS, the His-AKIN10 and GST-TICzj sample was not able to determining the phosphorylation sites on TICzj protein fragment (data not shown). As such, I needed to enhance the interaction and phosphorylation between AKIN10 and TIC fragments. Previous research has proved that the No. 175 serine (S175) is the autophosphorylation sites of AKIN10 on T-loop (t-loop); if changed this serine into aspartic to simulate the phosphorylation condition, the kinase may have more activity to phosphorylate the substrate. Next, I use the quick-change method as performed in chapter 2.2.3 to change the S175 into aspartic; this construct was named as AKIN10-S175D and the generated protein was named as His-AKIN10-S175D (Figure 3.3.2-1).

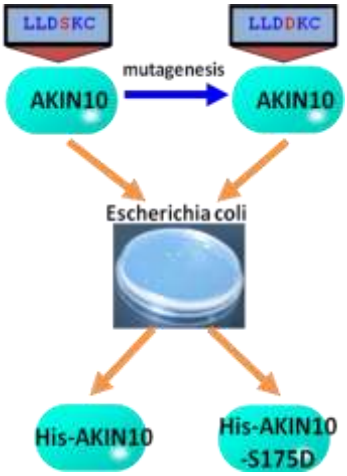


Figure 3.3.3-1 Protocol of making the construct of No.175 Serine-to-Aspartic mutation on AKIN10 full lengthe control.

As the same protocol of the kinase reaction for His-AKIN10 and GST-TICzj, the kinase reaction for His-AKIN10-S175D and GST-TICzj was incubated together with no radiation activity ATP for 2 hours. Next, I used trypsin to digested the sample, and the tryptic digested peptides from in vitro phosphorylation assay of His-AKIN10-S175D and GST-TICzj were subjected to MS analysis; the MS system was operated by Dr. Iris Finkemeier lab. I identified a total of TICzj phosphorylated by AKIN10 in vitro. Thus, the phosphorylation sites were determined. Tryptic digestion of TICzj yields the diagnostic peptide, VESSSGFRSDGEGAK, which includes S466 and for determining phosphorylation (Figure 2.3.3-2). The expected m/z for the nonphosphorylated peaks of VESSSGFRSDGEGAK is from 147.11270 to 599.27618 (Figure 3.3.2-2), the expected m/z of the phosphorylated peaks is 633.78955 (No.467 serine) and 698.31055 (No.466 serine) (Figure 3.3.2-2). However, in the AKIN10 and TICzj sample, the 633.78955 m/z peak was only has 0.8% phosphorylation possibility (calculated by ProteomeDiscoverer) (Figure 3.3.2-2), otherwise, the 738.29858 m/z peak was nonphosphorylated peak, this peak was the No.466 serine without the serine hydroxyl and this hydroxyl was at the phosphorylated position. This result also proved that No.466 serine was phosphorylated (Figure 3.3.2-2).

Finally, from the analysis results, the result displayed that only No.466 serine (S466) on TICzj could be the TIC's phosphorylation site for AKIN10.

3.3.4 No.466 serine on TIC is the phosphorylation site in vivo

With the result of the MS analysis, S466 could be TIC's phosphorylation site for AKIN10 in vitro. This result is important to demonstrated the mechanism of regulation between AKIN10 and TIC, but there is still a need to proved that TIC has the same phosphorylation sites of AKIN10 *in vivo*. To gain more information of this biochemical incident and explain more mechanism of regulation between AKIN10 and TIC, I wanted to detect the TIC phosphorylation sites in vivo. As TIC is a nuclear protein, I decided to use the MS analysis for detect the phosphorylation condition for nuclear protein in Col wild type seedlings.

To identify the phosphorylation sites of TIC *in vivo*, I obtained the cell nuclear protein (nucleoprotein) of Col wild type Arabidopsis, which was extracted. The Col wild type seedlings were germinated on MS1 plates and grown for 1 week, then harvested the total seedlings. The nucleoprotein was extracted by the protocol in material and method part in this thesis.

The tryptic digested peptides from the total nucleoprotein subjected to MS analysis. Thus, the phosphorylation sites were determined. Tryptic digestion of TIC protein yields the diagnostic peptide, VESSSGFR, which includes S466 and for determining phosphorylation. The analysis results displayed that S466 had 99.5% phosphorylation possibility, but S467 only had 0.5% phosphorylation possibility (the result was analysis by ProteomeDiscoverer) (Figure 2.3.3-1). The indication of phosphorylation of this MS analysis of nucleoprotein was similar to the MS analysis of AKIN10 and TICzj kinase reaction sample. The analysis results displayed that No.466 serine (S466) was also an *in vivo* phosphorylation site of TIC.

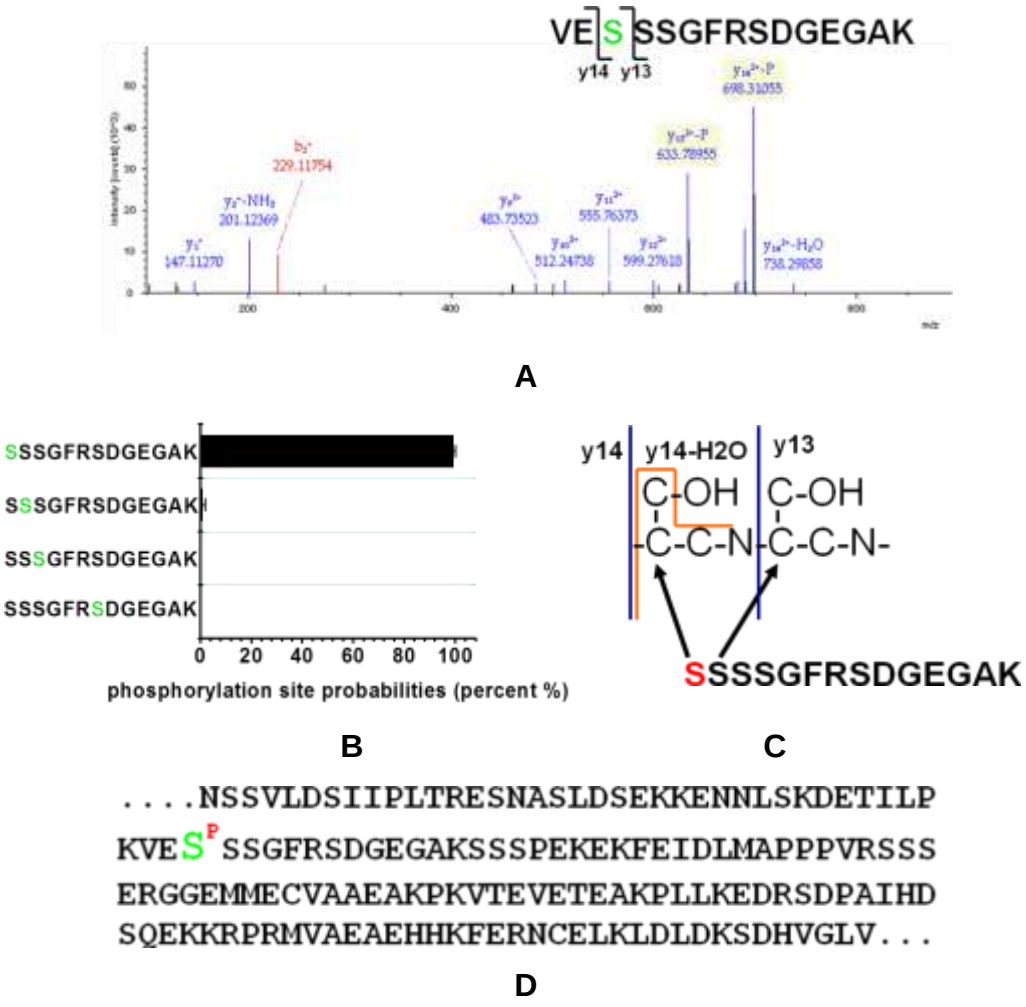


Figure 3.3.2-1 The MS analysis result of the phosphorylation sites of TICzj for AKIN10 in vitro

A. The bar graph of the digested peptide of VESSSGFRSDGEGAK, the peak y13 was displayed cleavage of SSGFRSDGEGAK and y14 was cleavage of SSSGFRSDGEGAK.

B. The phosphorylation site probabilities of each serine on fragment VESSSGFRSDGEGAK, the S466 displayed 99.2% probabilities, S467 only 0.8% and other serine had noon, the result was analysis by ProteomeDiscoverer.

C. Description of the difference between cleavage of y14 and y14-H2O, there is a carbon atom with alcohol hydroxyl connected with chiral carbon on serine, that alcohol hydroxyl was removed in y14-H2O. Due to the phoshporylation was on the hydroxyl, the y14-H2O cleavage wasn't detected any phoshporylation.

D. The localization of phoshporylation site for AKIN10 (S466) on TIC.

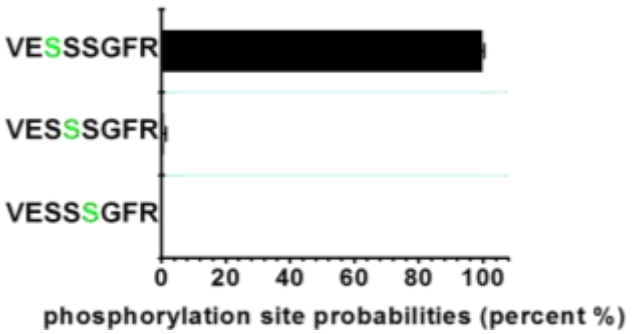


Figure 2.3.3-1 The phosphorylation site probabilities of each serine on fragment VESSSGFR in TIC from the MS analysis of the nucleoprotein of Col wild type in vivo, the S466 displayed 99.5% probabilities, S467 only 0.5%, the result was analysis by ProteomeDiscoverer.

3.3.5 Establish of TIC phoshorylation site serine to alanine direction mutants

S466 displayed a strong probability as TIC's phosphorylation site for AKIN10. This result demonstrates the mechanism of regulation between AKIN10 and TIC, but further studies were needed to show the interaction and phosphorylation between AKIN10 and TIC as an important regulation for the circadian clock..

To examine the effect of TIC in the regulation of the circadian clock under the phoshorylation from AKIN10, I prepared the construct of TIC full length with the mutation of No.466 serine to alanine, which was used mutagenesis technique. I named this construct as TIC-S466A. To include more genetic backgrounds of Arabidopsis, I transform this construct to *tic*-1 mutant which was Ws background. Floral dip transformation was not possible on *tic*-2 mutants of Col back ground. As such, I introduced the construct into Col wild type by crossing.

3.3.6 Establish of the construct of TIC with deletion of No. 479 to 481

Due to the result of the MS analysis in vitro, it was indicated that the S466 could be the TIC's phosphorylation site for AKIN10. It is worth noting there are few small missed cleavages in MS analysis result; in this case, the protein sequence was digested after arginine or lysine by tryptic digestion, the SSSPEK peptide was missed

because of the side was too small. Otherwise in the Col wild type nucleoprotein MS analysis result displayed that 33.3% phosphorylation probability on the 3 consecutive serine of the peptide of SSSPEKEKFEIDLMAPPPVR for each serine, it still need to detect that these 3 serine's phosphorylation condition.

To detect if there are more phosphorylation sites on TIC for AKIN10, following the MS analysis result of the possible phoshporylation sites on peptide SSSPEKEKFEIDLMAPPPVR, I made the construct with TIC full length and use the PIPE PCR delete the DNA sequence which coded this 3 consecutive serine to generate the TIC protein fragment without this 3 serine for detect these serine was phoshporylated or not. I named this construct as TIC-Δ479- 481.

3.3.7 Discussion

As with published results and results in this thesis, TIC is an important regulator for clock activity in clock input pathway and the circadian period and amplitude was changed in *tic*, AKIN10, and *tic*-2/*AKIN10*. Thus, the TIC protein also regulated clock-driven on the input pathway of clock [11-12]. In this study, the result indicated that AKIN10 could be upstream of TIC in the input regulation pathway for circadian clock and function through AKIN10-TIC interaction and phoshorylation.

However, the relationship between TIC and AKIN10 has not been clearly defined. In this study, I generated TICzj fragment, AKIN10 and AKIN10-S175D protein *in vitro*, thus, the comparable positive result as the yeast-two-hybrid was showed in the in vitro pull down experiment with TICzj fragment and AKIN10, these results indicated that TIC could be an interactor of AKIN10. As AKIN10 is a kinase and has important function in the phosphorylation of other proteins, I tested if these two protein has phosphorylation or not, and found that AKIN10 could phosphorylate TIC in vitro. These results indicated that AKIN10 could interact and phosphorylate TIC in vitro, and that TIC was a possible interactor and kinase phosphorylation substrate of AKIN10 in vivo. For mapping the phosphorylation site on TIC for AKIN10, I used MS to analyze the TICzj and AKIN10 kinase reaction sample; the result indicated that

S466 could be phosphorylation site of TIC for AKIN10. As the previous result showed that AKIN10 could be upstream of TIC on the input pathway of circadian clock, this study contributed more information for the mechanism between TIC and AKIN10.

For the biochemical mechanisms, the protein phosphorylation influences the activity of substrate proteins through diverse mechanisms, such as modulation of their nucleo-cytoplasmic distributions, DNA-binding properties, and protein stabilities and modification of their interactions with other regulatory proteins. As a previous study of TIC displayed that TIC is a nucleoprotein, that indicated that AKIN10 does not change the nucleo-cytoplasmic distributions of TIC. The previous research also indicated that TIC was suggested to be involved in the protein-degradation process, as depletion of its binding partner MYC2 was promoted by TIC [116]. Furthermore, proteasome degradation is very important in the mechanism of needless protein degradation, and the phosphorylation modification is possible to change the proteasomes degradation condition (MAPK). For this study and plus the previous result in our lab, TIC could be a multiple protein interactor for other proteins, if the phoshporylation from AKIN10 could regulate TIC's degradation. Thus, the interactions of proteins on TIC include the potential TIC downstream circadian clock regulator, these also could be regulated by AKIN10 through its phosphorylation for TIC. The phosphorylation site is very important for kinase research. In this work, the result of in vitro kinase assay indicated that the No. 466 Serine on TIC was the phosphorylation site for AKIN10, but the No. 479 to 481 three Serine were showed that 33% phosphorylation possibility on MS, this part still needs further investigation to detect if there are phosphorylation sites on these serine.

For the phenotypes of these plant lines, *tic*-2, *tic*-2/*AKIN10* all showed short period phenotype compared with wild type and *AKIN10* displayed a long period phenotype. This indicated that AKIN10 was the upstream regulator of TIC in the input pathway of circadian clock. In this study, the phosphorylation between AKIN10 and

TIC in vitro was found, and the potential of TIC's phosphorylation sites with AKIN10 was also identified from the mapping with MS analysis. These results contribute to explain AKIN10 and TIC complex formation and its regulatory mechanism to the circadian clock.

Additionally, it is noteworthy that the molecular nature of the *akin10* mutant was still controversial. The *akin10* mutant is a null mutant through AKIN10 gene expression study and immunological detection of AKIN10 proteins was reported [120]. In this study, the *tic*-2, *tic*-2/*AKIN10*, AKIN10 lines were included in the description of the circadian period phenotype and explain the up and down stream relationship between AKIN10 and TIC in circadian clock pathway, the *akin10* was not used. There are many energy-consuming developmental processes in plants. Therefore, it is not surprising that the circadian clock was closely regulated

As the previous results displayed that SnRK1 plays a fundamental function on the carbon availability for the plant developmental process [121]. SnRK1 members coordinate varying regulation in transcriptional networks, which are involved in several catabolism but made inhibition of anabolism to sustain cellular energy homeostasis under stress [77,105,121]. Only a few substrates of SnRK1members have been identified in the various cellular responses.

With the results from the study and our lab's previous results, the interaction between TICzj fragment and AKIN10 in vitro was found. In this work, I displayed the phosphorylation happened between these two proteins (Figure 3.2.2-1). The kinase energy functional process of AKIN10 and its interaction of TIC *in vivo* indicated that TIC was an important connector between the clock and energy transformation. As the investigation of the phosphorylation site in TIC for AKIN10, the MS result was displayed the No.466 Ser was the phosphorylation site in TIC for AKIN10 in vivo. In conclusion, the result of this work indicated that the serine/threonine-specific kinase AKIN10 and its target TIC constitute a sugar metabolism-mediated circadian clock path way. On the basis of circadian period description of *tic*-2 mutants and transgenic

plants over-express AKIN10 genes in *tic*-2 and biochemical examination of AKIN10-mediated phosphorylation of TIC, I suggest that the AKIN10 path way senses fluctuations in sugar metabolism, energy transformation and integrates the metabolic signals into the TIC-mediated gene regulatory regulation of circadian clock.

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